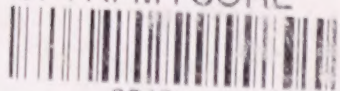


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BIOLOGICAL ANTAGONISM

The Theory of Biological Relativity

BIOLOGICAL ANTAGONISM

THE THEORY OF BIOLOGICAL RELATIVITY

By GUSTAV J. MARTIN, Sc.D.

Research Director

The National Drug Company

Philadelphia



1951

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Philadelphia

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PREFACE

At rare intervals into the systems of biological research come concepts which are intriguing to the scientific mind. These concepts are frequently of such simplicity as to cause wonder at their delayed arrival. Then, on closer examination, it is seen that a period of growth extending over many years led to the ultimate fully formed structure. Such a concept is the one of structural displacement, which rightly has been called "The Rational Approach to Chemotherapy."

To the immunologists goes credit for the first recognition of the value of the theory. Ehrlich applied it in his famed "Lock and Key" analogy. A more rewarding and stimulating approach to immunology had not been suggested before his time nor has one been recorded since. An entire world—the world of the antigen and antibody—grew up around this "Lock and Key" approach.

Enzyme chemists soon took up the new concept and developed it into a fundamental structure. To the embarrassment of the biochemist and pharmacologist alike, it must be admitted that research in these fields failed to recognize the merits of the metabolite analogue approach until much later.

The renaissance of the concept came with the work of Woods in England in 1940. Its popularity grew until 1947 when many early enthusiasts began to abandon the cause. The concept remains a storehouse of potential chemotherapeutic and pharmacological agents.

The purpose motivating the preparation of this summary of knowledge in the field of displacement is a belief that in no single instance of specific displacement has a thorough job been done, and that such work, properly undertaken, will lead to discoveries of chemotherapeutic agents of great value in medical science. The author feels that a reference work on the subject will facilitate the entry of others into the field and thus aid in causing the concept to be formed into a scientific structure of great practicality.

It is to be assumed that the first attempt at an outline of a concept of such great scope may result in a failure to cover adequately all works of merit. Future revisions of this volume are contemplated and all criticisms and suggestions will be gratefully received.

The author wishes to express his sincere thanks to those individuals whose cooperative spirit lightened the burdens associated with the prepa-

ration of this manuscript. He is grateful to Drs. Robert Raffauf, Werner Boehme, and J. Morton Beiler for the time they spent checking technical accuracy. Further, acknowledgment is made of the fine work done by Miss Doris Testardi and Mrs. Katherine Delsordo in the typing and proofreading of the book. He wishes to thank Mrs. Patricia Martin for her constant help and encouragement. Finally, he is deeply indebted to Mr. A. B. Collins for his sympathetic understanding and support of this work.

GUSTAV J. MARTIN

Philadelphia
August 1951

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Chapter 1

ENZYMATIC INHIBITION BY CHEMICALS STRUCTURALLY RELATED TO THE NATURAL SUBSTRATE

1. Specific Action of Enzymes.
2. Kinetics of Enzyme Action.
3. Kinetics of Enzyme Inhibition.
4. Succinic Dehydrogenase, Lactic Acid Dehydrogenase.
5. Acetate Metabolism; Fluoroacetate and Related Molecules.
6. Lipases, Liver Esterase, and Pancreatic Lipase.
7. Urease.
8. Certain Enzymes Involved in Carbohydrate Metabolism.
9. Miscellaneous.
10. Recapitulation.

In opening a review of metabolite analogues with a discussion of enzymatic inhibition by chemicals structurally related to the natural substrate, the purpose is to cover briefly specificity and kinetics. A consideration of the kinetics of enzymatic inhibition will serve to focus attention on the fact that the subject covers competitive and noncompetitive inhibitors, as well as those not yet classified.

Many of the displacing agents to be considered later in this book function by virtue of similarity in structure to substrate; their exclusion from this chapter is in the interests of organization and is not based upon any distinction in basic mechanism.

Specific Action of Enzymes

Enzymes possess absolute, stereochemical, and relative specificity. As an example, carboxypeptidase has absolute specificity in that it will not attack carbohydrates or fats; stereochemical specificity in that it will not hydrolyze synthetic peptides whose terminal amino acids are of the D-configuration, although the corresponding L-compounds are sensitive; and relative specificity in that it hydrolyzes carbobenzoxyglycyl- β -2-thienylalanine at only one-half the rate for the benzene relative. The study of metabolite analogues is based upon the existence of a high degree of selectivity. In fact, such study is also an approach to the determination of the degree of enzymatic

specificity. Examples of enzymatic selectivity are presented in the textbook of enzymology by Sumner and Somers (1947).

Kinetics of Enzyme Action

The inhibitory power of any given antimetabolite will be a direct function of its effect on the rate of an enzymatic reaction. Specifically, potency will be a function of the capacity of a chemical to reduce the available products of an enzyme mechanism. It must be kept in mind that no enzyme is "an island unto itself" and therefore the *in vivo* activity will be a function of the inhibitory action and, in some cases, stimulatory effect of a compound on a series of enzymes.

The law of mass action states that the rate of a chemical reaction is proportional to the active masses of the reacting substances. This holds for catalyzed reactions. The rate of a reaction is the speed at which the concentration of any given reacting molecule is changing at a given instant. With enzymatic reactions, there are optimal pH and temperature values which, unless specified, are assumed to be controlled and constant.

The speed or velocity of an enzymatic reaction is initially constant, then declines progressively with time due to the exhaustion of substrate, accumulation of end-products, etc. In general, in all considerations of antimetabolites, we are concerned with this initial and constant reaction rate.

For purposes of clarification, it seems wise to review the mathematical formulations for the kinetics of ordinary reactions.

First order reactions are those in which the rate of decomposition is directly proportional to concentration. The mathematical expression is:

$$-\frac{dc}{dt} = kc$$

where c is the concentration of the material, k is a proportionality factor, and $-\frac{dc}{dt}$ is the rate of change. This equation may be modified as follows:

$$k = \frac{2.303}{t} \log \frac{a}{a-x}$$

where a is the initial concentration, x is the amount reacting in time t , and $a - x$ is the concentration remaining after time t . k is the velocity constant.

Second order reactions involve two molecules and depend upon collision frequency. The mathematical expression is:

$$\frac{dx}{dt} = k(a-x)(b-x)$$

where a and b represent initial molar concentrations, x denotes amounts changed in time t . On integration of the equation, taking into consideration that $x = 0$ when $t = 0$, and that $x = x$ when $t = t$, it is seen that:

$$k = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)}$$

where k is the reaction constant.

The mathematical expression of a zero order reaction is:

$$-\frac{dc}{dt} = k = \text{rate of reaction}$$

Here the rate of reaction is constant over a period of time.

In enzymatic reactions, another factor—concentration of enzyme—is added. Such reactions are generally of zero or first order; however, it should be kept in mind that the order of an enzymatic reaction may change as it proceeds. Enzymatic reactions of zero order are expressed as:

$$-\frac{dC_a}{dt} = kC_e$$

where C_a is the concentration of the reactant; C_e is the concentration of the enzyme.

The first order enzymatic reaction is expressed as:

$$-\frac{dC_a}{dt} = kC_aC_e = \text{reaction rate}$$

and if C_e is constant over a short period of time, the reaction has the characteristics of an ordinary first order reaction and can be written:

$$kC_e = K' = \frac{2.303}{t} \log \frac{a}{(a-x)}$$

a and x have same significance as is given above.

Another form of this equation which is the most practical for purposes of calculation is:

$$K' = \frac{2.303}{t_2 - t_1} \log \frac{C_1}{C_2}$$

where C_1 and C_2 are substrate concentrations at times t_1 and t_2 .

Second order enzymatic reactions are expressed as:

$$-\frac{dC_a}{dt} = -\frac{dC_b}{dt} = kC_eC_aC_b = \text{rate of reaction}$$

C_e , C_a , and C_b respectively are the concentrations at time t of enzyme and the reactants a and b of the second order reaction.

Zero order enzymatic reactions are those in which the concentration of the substrate is high compared to the amount of enzyme employed. This assures a constant concentration of the enzyme-substrate complex which is the reactant in this type or order of reaction. The enzyme-substrate complex is an assumption but for all practical purposes seems in order.

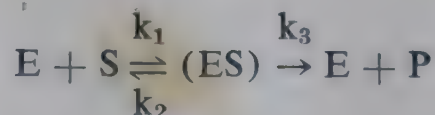
First order reactions vary in rate as the substrate concentration decreases and the product formed increases in amount. The rate will slow as time progresses. The action of hydrolases in dilute solution is a good example.

Whether or not second order enzymatic reactions occur is open to question. If they do, the speed varies with the product of the concentrations of the reactants.

The order of a reaction is determined by plotting graphically different functions of concentration of reactant against time and determining which one gives a straight line. If $\log c$ against time gives a straight line the reaction is of the first order, which means that the time taken for a given fraction to react is independent of the initial concentration. A second order reaction would give a straight line by plotting $\log (a - x)/(b - x)$ against time or when the initial concentrations of the reactants are equal, by plotting $1/c$ against time. In a zero order reaction, plotting c directly against time would give a straight line, indicating a constant rate of reaction over at least a short interval of time.

As the order of enzymatic reactions may shift, it is advisable from a practical standpoint to consider only the initial rate. At this stage, concentrations of reactants and enzyme are more stable and the reverse phase which is a characteristic of most enzymatic reactions has not yet come to play a significant part. Enzyme concentrations in these mathematical formulations are expressed in terms of their activity.

Neurath and Schwert (1950) have presented an analysis based on kinetics of inhibitor action which considers the concentration of the enzyme-substrate complex and therefore differs slightly from the equations presented above. The formulation for enzymatic action represents an extension of the concepts of Michaelis and Menten (1913) and is expressed as follows:



E = free enzyme. S = free substrate. ES = enzyme-substrate complex. P = reaction products. k_1 , k_2 , and k_3 = rate constants.

The rate of the overall reaction is represented as:

$$d(p + a) / dt = - k_3 p$$

where p and a represent concentrations of enzyme-substrate complex and free substrate. For a zero order reaction this reduces to a modified form the equation for the zero order enzymatic reaction given above, namely:

$$- da/dt = k_3 p$$

In other words, the rate is proportional to the concentration of the enzyme-substrate complex. In the system under consideration, the concentration of

the enzyme-substrate is constant which leads to the mathematical formulation:

$$\frac{k_2 + k_3}{k_1} = \frac{(e - p)a}{p} = \frac{(E)(S)}{(ES)} = K_m$$

From this equation it is possible to derive an expression for reaction velocity in terms of enzyme and substrate concentrations:

$$v = -da/dt = \frac{k_3ae}{K_m + a}$$

Still another mode of presentation of velocity rate is that in which all of the enzyme is bound in an enzyme-substrate complex. This then represents a maximum velocity, V_{max} , and the equation becomes:

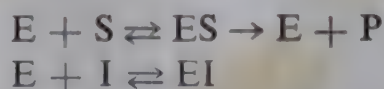
$$v = \frac{V_{max} a}{K_m + a}$$

A linear relation between v and a is obtained from the equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \left(\frac{1}{a}\right) + \frac{1}{V_{max}}$$

Kinetics of Enzyme Inhibition

For presentation purposes, Neurath and Schwert (1950) presented a mathematical kinetic representation of the inhibition of proteolytic reactions. For the competitive type of inhibition, it is assumed that both inhibitor and nutritive compete for the same reaction site on an enzyme surface. This can be represented as follows:



I and EI indicate inhibitor and enzyme-inhibitor complex concentration. The degree of inhibition depends on the concentrations of ES and I and can be expressed as:

$$\frac{1}{v_i} = \frac{1}{V_{max}} + \left[1 + \frac{(I)}{K_i} \right] \frac{K_m}{V_{max}} \frac{1}{a}$$

V_i = initial velocity in the presence of the inhibitor.

K_i = dissociation constant of the enzyme-inhibitor complex.

I = concentration of inhibitor.

The noncompetitive type of inhibition assumes reaction of inhibitor with catalytically inactive sites on the enzyme surface and is expressed as follows:



and mathematically as:

$$\frac{1}{v_i} = \left[1 + \frac{(I)}{K_i} \right] \left[\frac{1}{V_{\max}^i} + \frac{K_m}{V_{\max}^i} \frac{1}{a} \right]$$

$V_{\max}^i$ = maximum velocity in the presence of an inhibitor.

There are instances in which the action of an inhibitor may be non-competitive at one concentration and become competitive as the concentration increases and the inhibitor begins to be bound at the catalytically active enzyme surface site.

Hunter and Downs (1945) presented a mathematical expression of the kinetics of inhibitors which assumed a negligible fraction of total inhibitor present combined with enzyme. For a noncompetitive system, the formulation was:

$$I \frac{a}{1 - a} = K_I$$

K_I = dissociation constant of enzyme inhibitor complex.

I = concentration of inhibitor.

a = fractional activity of enzyme.

This equation shows that with given enzyme and inhibitor concentrations, the fractional activity is constant and independent of substrate concentration. The controlling factor is the degree of dissociation of the enzyme-inhibitor complex.

For competitive inhibition, the Hunter and Downs equation is:

$$I \cdot \frac{a}{1 - a} = K_I + \frac{K_I}{K_s} \cdot S$$

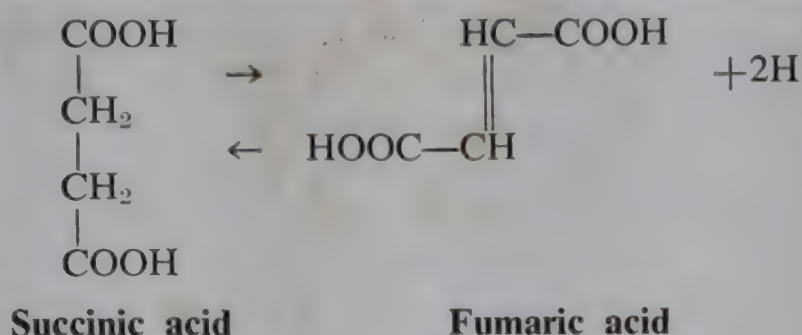
S = substrate concentration.

K_s = dissociation constant of enzyme-substrate complex.

The equation shows that the extent of inhibition is proportional to the relative values of the dissociation constants of the enzyme-inhibitor and enzyme-substrate complexes and to inhibitor and substrate concentrations. The effectiveness of a given competitive inhibitor depends upon its concentration and relative affinity for the enzyme as compared to the substrate.

Succinic Dehydrogenase, Lactic Acid Dehydrogenase

Succinic dehydrogenase is one of a group of enzymes possessing the ability to reduce cytochrome C. Recent evidence indicates that this might not be a direct mechanism. The enzyme converts succinic acid to fumaric acid provided a hydrogen acceptor is present. Under aerobic conditions the hydrogen goes to oxygen by the action of cytochrome-cytochrome-oxidase.



This transformation is part of the tricarboxylic acid (Krebs) cycle in which oxalacetic acid condenses with acetic acid in a chain of reactions which eventually give rise to α -ketoglutaric acid and carbon dioxide. α -Ketoglutaric acid is then decarboxylated to succinic acid and it is at this point that succinic dehydrogenase functions in bringing about the next step to fumaric acid. The final phase is regeneration of oxalacetic acid which partakes in another cycle.

Quastel and Wooldridge (1928) first studied the inhibition of succinic dehydrogenase by substrates structurally related to succinic acid. Malonic acid, which is the lower homologue of succinic acid, blocked the action of the enzyme by preventing access of succinic acid to the reaction site. The mechanism was that of purely competitive inhibition as demonstrated by Hopkins et al. (1938) who found that adequate succinic acid completely overcame the antimetabolite action. In addition to malonic acid, Quastel and Wooldridge found an entire series of carboxylic acids to be effective inhibitors (Fig. 1).

In the inhibition of succinic dehydrogenase by malonic acid, the affinity of antimetabolite and enzyme is so great that the inhibitor/substrate ratio for 50 per cent inhibition is 1/50 (Potter and DuBois, 1943). This is an exceptional case and represents one of the few instances in which K_I is lower than K_S .

Krebs and his coworkers (1937, 1940, 1940a, 1940b) used malonic acid as an important tool in establishing the citric acid cycle. They found that fumaric acid removes the malonate inhibition of pyruvate oxidation; succinic acid accumulated under the conditions of the experiment. Again, in the presence of malonic acid, they found that fumaric disappears and succinic accumulates even if no pyruvate is added.

Other compounds known to inhibit succinic acid dehydrogenase are: oxalic, adipic, aspartic, malic, fumaric, and oxalacetic acids (Bandhu, 1937; Potter and Elvehjem, 1937).

Of a series of alkylated malic, succinic, and malonic acid derivatives, Franke (1944) found that the alkylmalonic acids did not inhibit succinic dehydrogenase. The alkyl succinic acids from the octyl to the dodecyl compounds were inhibitors; the alkyl malic acids were not. In general, it would seem that the carbon alkyl acids of this type are either inactive or weak

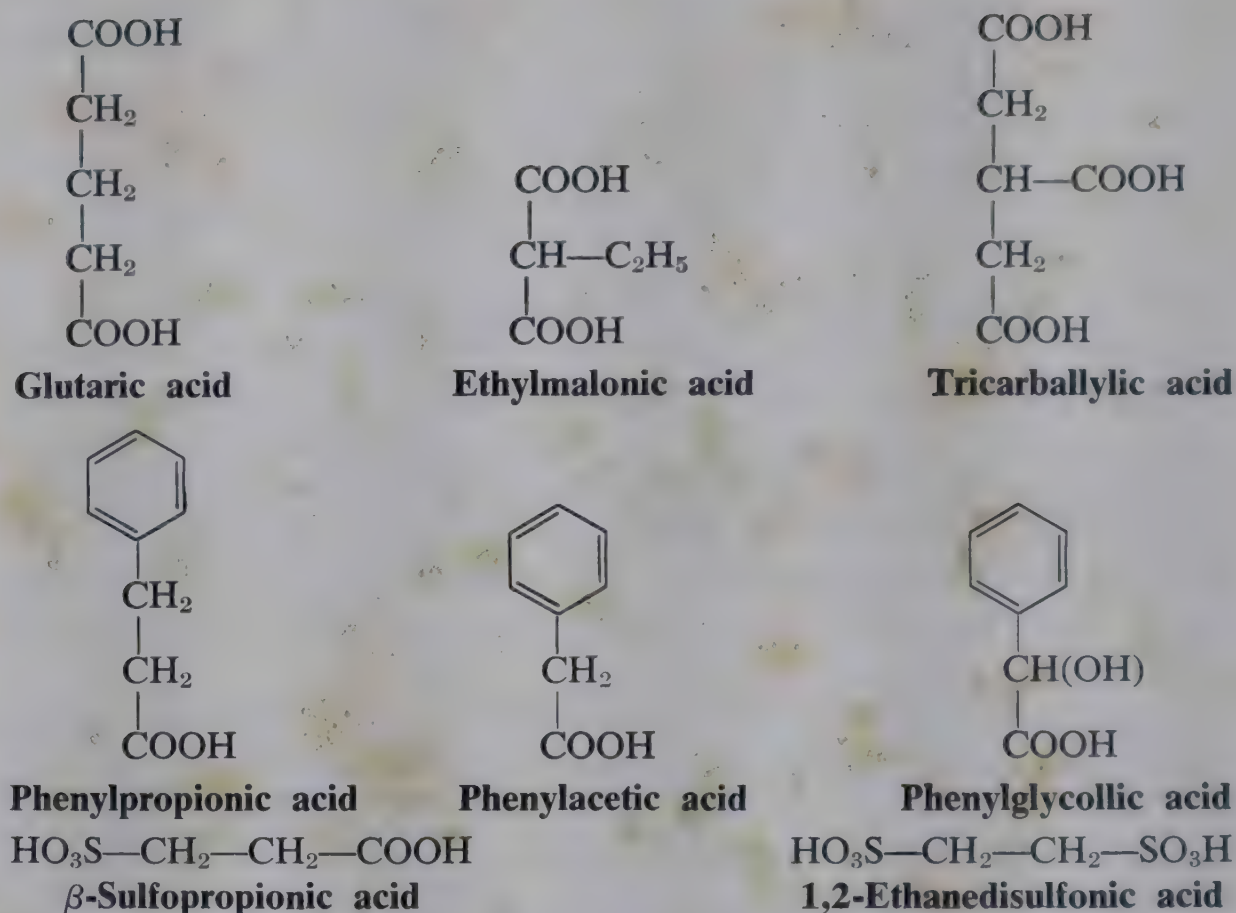


FIG. 1. Metabolite antagonists of succinic acid.

inhibitors. The sulfonic acid analogues of succinic acid— β -sulfopropionic acid and 1,2-ethanedisulfonic acid—are both effective antimetabolites with a potency roughly equivalent to that of malonic acid (Klotz and Tietze, 1947). The competitive or noncompetitive nature of the action was not determined but it seems probable that as with so many other sulfonic acid analogues the activity would be nonspecific.

Recently, Pardee and Potter (1949) discovered the blockage of oxalacetate oxidation by malonate, a phenomenon partially dependent upon the concentration of magnesium ions. The formation of a complex of malonate with free and bound magnesium was offered in explanation of the findings. This would probably be a chelation mechanism, which seems to assume more and more importance in biological relativity. They do not imply that magnesium chelation (complex formation) underlies the entire blockage process but they do regard it as an important factor.

Again, the succinoxidase system has been employed by Ackermann and Potter (1949) to extend the differential metabolite requirement theory (Martin, 1944) to the equivalent of a differential enzyme concentration-inhibitor concept. This basic idea, a manifestation of the biological relativity theory, proposes that the effect of a given inhibitor *in vivo* will be greatest for the tissue containing the inhibited enzyme in the lowest concentration. Ackermann and Potter use as an example of the application of

this concept the production of alloxan diabetes (see purine and pyrimidine section, p. 382). They point out that alloxan is a general sulfhydryl inhibitor and that selective action against the insulin-producing cells is therefore unlikely. The only logical alternative would be that these cells are more vulnerable because quantitatively they lack a vital enzyme, plentiful in other tissues.

The main point made in their presentation is that the so-called irreversible enzyme inhibitors, such as copper in the case of succinic dehydrogenase, exert their action in proportion to the concentration of the enzyme. In a complex in vivo system, the effect of the irreversible inhibitors will be a function not only of the enzyme concentration but also of the concentration of other substances which will combine with the inhibitor. This is the basis of the observed reversal of the action of copper on succinoxidase by glutathione.

There have been many other studies of succinic acid analogues and comparatively few of them can be discussed in the interests of brevity, but there is one—namely, trans-1,2-cyclopentanedicarboxylic acid—which brings into consideration another factor in the study of displacement. Seaman and Houlihan (1950) have reported that this compound increases the permeability of the membrane of *Tetrahymena geleii* to succinate. Normally, the membrane is impermeable to succinic acid. The in vivo action of this molecule, in itself an inhibitor of the oxidation of succinate, should differ materially from that of a compound capable of inhibiting in vitro but without a similar action on permeability. The alteration of membrane permeability by metabolite analogues may well be a function of their inhibitory capacity or it might be dissociated therefrom. Research along these lines offers hope for discoveries in the field of membrane permeability.

Another study of general importance bearing on this subject is that of Schulman and Armstrong (1949, 1949a). They tested a series of compounds of the type $X(CH_2)_nY$ for activating or inhibiting power in the decolorization of methylene blue by yeast cells or by succinic dehydrogenase. With this enzyme, the order of decreasing inhibitory power is as follows:

$X, Y; -CH(NH_2), -NH^+; H, CH(NH_2)NH^+; H, NH_3^+; H, SO_4^-; H, CO_3^-; H, NMe_3^+; H, SO_3^-; H, \text{bile salt}.$

The same compounds activated the yeast cell systems and the activation probably is due to the increased permeability of the cell membrane for the methylene blue. Here, again, the factor of membrane permeability plays a vital role.

These investigators (Schulman and Armstrong, 1949a) extended their consideration of the permeability factor and report that only compounds with one long substituted chain in a series of alkyl substituted succinic acids

and half esters activate the decolorization of methylene blue by yeast cells.

Finally, the noncompetitive inhibition of succinic dehydrogenase by mononucleotides (Zittle, 1946) appears to confuse the picture until it is viewed in the light of the energy transfer of the dehydrogenase mechanism to high potential phosphate bonds such as those of adenosine triphosphate, phosphocreatine, and enol phosphate (Colowick et al., 1941). This cross link on an enzymatic system may represent another type of inhibition, in that the mononucleotide competitively inhibits a system which receives energy from the dehydrogenase; blocking this energy transfer retards the dehydrogenase.

Coincident with their work on succinic dehydrogenase, Quastel and Wooldridge (1928) studied the inhibition of the dehydrogenation of lactate to pyruvate (lactic acid dehydrogenase) and found that the reaction was blocked by an entire series of molecules, including α -hydroxybutyric, glyceric, mandelic, glyoxylic, and oxalic acids. Malonic acid was subsequently found to exert a similar action (Das, 1937).

Acetate Metabolism; Fluoroacetate and Related Molecules

One of the first things that comes to mind in a consideration of fluoroacetate is the biological activity of its relative, iodoacetate. Iodoacetate is known to inactivate or inhibit choline acetylase (Nachmansohn and Machado, 1943), papain (Fruton and Bergmann, 1940), yeast alcohol dehydrogenase (Dixon, 1937), isocitric dehydrogenase, enolase (Meyerhof and Kiessling, 1935), 1,3-diphosphoglyceric aldehyde dehydrogenase (Adler et al., 1938), and aldehyde mutase (Dixon, 1938-39). Its function seems to be that of reacting with sulfhydryl groups of the enzyme protein, causing inactivation. Its effect is therefore nonspecific and it will block any enzyme requiring intact —SH for activity. This is mentioned to emphasize the fact that there is no biochemical, physiological, or pharmacological similarity between iodoacetate and fluoroacetate action.

Attention should be directed to the basic mechanism of action of fluoroacetate, which is that of interference with acetate metabolism by prevention of the formation of an "active" acetate (Bartlett and Barron, 1947). Furthermore, fluoroacetate has been isolated from the South African plant, *Dichapetalum cymosum*, by Marias (1944). The plant, which was known locally as "Gifblaar," had long been recognized as toxic to livestock. This is another example of a "synthetic" antimetabolite which is also found in nature.

The studies that have established the displacer activity of monofluoroacetate are too numerous to be reviewed here. An excellent and detailed summary of this information is contained in an article by Chenoweth (1949). It is indicated, however, that some of the background work be considered.

Many test systems have been used in studies of fluoroacetate. One of these, which is pharmacologically significant in addition to being a reflection of basic mechanism, involves the use of intestinal smooth muscle. Weeks et al. (1950) found that both sodium dehydroacetate and fluoroacetate interfere with the maintenance of spontaneous contractility in intestinal strips. The toxic effect of both displacers was counteracted by glucose and sodium acetate. Similar findings were simultaneously reported by Farah et al. (1950). Pyruvate and some of the even numbered carbon fatty acids, as well as acetate, were able to sustain contractions in the normal muscle but failed when sodium fluoroacetate was added. There was a quantitative relationship between the concentrations of sodium acetate and fluoroacetate which would produce a certain degree of inhibition, a fact which was interpreted to indicate competitive mutual antagonism. Another facet of their observations concerned the fluoroacetate sensitive and resistant contractions. The proposed explanation was that two pathways of energy supply for contraction exist; the sensitive reaction via pyruvate and acetate, the other via a pathway involving neither the Krebs cycle nor the cytochrome system. It seems probable that maintenance of contractility might be effected through the addition to the system of phosphocreatine or adenosine triphosphate as Colowick et al. (1949) have demonstrated a failure of oxidative resynthesis of phosphocreatine by frog muscle poisoned by methyl fluoroacetate.

In the isolated heart, similar effects of fluoroacetate are noted. Either acetate or glucose in the perfusate will maintain contraction and protect the rabbit and monkey heart against fluoroacetate (Chenoweth, 1949). The molar ratio for protection was approximately unity. Pyruvate is less effective than acetate as a biochemical antidote for fluoroacetate (Braun-Menendez et al., 1939).

The resting potential of frog nerve is preserved by the formation of pyruvate and its subsequent aerobic metabolism. Methylfluoroacetate blocks the recovery of resting potential of nerve in oxygen following anoxia (Shanes, 1949), an action prevented by sodium pyruvate. Sodium fluoroacetate is not active in isolated frog brain preparations (Brooks et al., 1949).

Mechanism studies of the action of monofluoroacetate in intact animals have revealed that sodium acetate is an effective antidote (Tourtellotte and Coon, 1949). Ethanol was also active, and a combination with acetic acid suggested synergism. Confirmation of this work came from Hutchens et al. (1949) and its subsequent extension revealed the effectiveness of other two carbon moieties, particularly glycerol monoacetate (Chenoweth et al., 1949a).

Studies of tissue slices, homogenates, and microorganisms have revealed that the basic action of fluoroacetate is essentially similar throughout. The

first such study was that of Bartlett and Barron (1947) who found that fluoroacetate had no effect on sulfhydryl enzymes. In the presence of the compound in an isolated tissue, acetate accumulated in the presence of pyruvic acid, the oxidation of fatty acids and the formation of acetoacetic acid was blocked, and carbohydrate synthesis from acetate or pyruvate was stopped. Acetylation reactions were not prevented when sulfanilamide, p-aminobenzoic acid, and choline were substrates. This is an important example of the selectivity of antimetabolites in the blockage of one channel of substrate metabolism with no effect on another. In 1948, Kalnitsky and Barron published the results of their extended studies. Using fresh rabbit kidney homogenates, they observed the inhibition by fluoroacetate and fluorobutyrate of the oxidation of caproic, acetic, butyric, pyruvic α -ketovaleric and α -ketocaproic acids, and glucose. Ethyl alcohol released the fluoroacetate inhibition of acetic acid oxidation.

Again, with microorganisms, fluoroacetate completely inhibits the oxidation of yeast (Kalnitsky and Barron, 1947). If the acetate is added before the inhibitor, there is no blockage. This is clear evidence supporting the competitive nature of the phenomenon. Neither the other halogen acetates nor trifluoroacetate had this activity. The inhibition occurs in the first step of acetate metabolism, as shown by inhibition of citric acid synthesis from acetate and the accumulation of acetate during the oxidation of glucose or ethanol. Black and Hutchens (1948) have studied the time element in the fluoroacetate-acetate system. If the inhibitor was added to yeast 25–30 minutes before the acetate, no significant oxygen uptake occurred until an extended induction period (approx. 3 hrs.) had elapsed. During this period, the addition to the system of ethanol, acetaldehyde, succinate, and succinic semialdehyde catalyzed the oxidation.

An observation such as that of Fitzgerald et al. (1949) on the inhibition by iodoacetate and fluoroacetate of the adaptive enzyme formation in *Mycobacterium lacticola* probably represents a nonspecific phenomenon, not associated with direct competitive displacement of acetate.

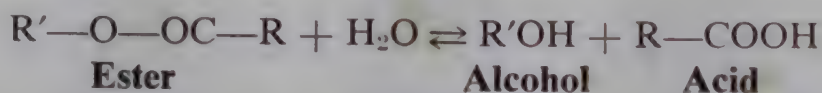
In addition to the nonspecific physiological activity, it is logical to expect certain related molecules to act by conversion to fluoroacetate. This is the case with fluoroethanol (Bartlett, 1949), which functions entirely by virtue of oxidation to the corresponding acid and in itself possesses no activity. In fact, Saunders (1947) concluded from the study of the toxicity of a series of homologous compounds of the type $F(CH_2)_nCOOR$ that only those members were toxic which could give rise to fluoroacetate by β -oxidation.

The pharmacological and toxicological characteristics of monofluoroacetate have been reviewed in detail by Chenoweth (1949) and it is clear that certain information presented in his review correlates with the hypothesis of Ackermann and Potter (1949) that any given antimetabolite will

function as a toxic agent in a specified system in accordance with the relative importance of the affected enzyme to that system. The toxicity of fluoroacetate varies with species from an LD_{50} of 0.06 mg./kg. in the dog to over 500 mg./kg. in a species of toad. Here, the experimental findings would indicate that the enzymatic mechanisms blocked by fluoroacetate were roughly 10,000 times more important to the dog than to the toad, a good working example of the theory of biological relativity. Again, the major point of attack on specific tissues varies; it may be the central nervous system, the heart, or both. The organ or tissue affected is that one to which the blocked enzymatic reactions are the most vital—the tissue containing the lowest concentration of the inhibited enzyme. It is interesting to speculate on the reason for cardiac manifestations in herbivorous animals, and central nervous system effects in carnivores.

Lipases, Liver Esterase, and Pancreatic Lipase

The lipases are a subgroup in the general classification, esterases. All esterases catalyze the reversible reaction:



The specificity of lipases has been the subject of study by Weinstein and Wynne (1935–36) who found that the initial rates of hydrolysis of some triglycerides have the following order: tripropionin > tributyrin > tricaproin > triacetin > trivalerin. While the pancreatic lipases will hydrolyze simple esters, liver esterase is relatively specific for these molecules, especially ethyl butyrate. Liver esterase will act on fats, but very slowly.

Murray and King (1930) first investigated the relative affinities of pairs of optically active secondary alcohols for liver esterase by observing the extent to which their presence inhibits the hydrolysis of ethyl butyrate or ethyl propionate. The L-forms of methyl-n-hexylcarbinol, methylphenylcarbinol, and methyl- β -phenylethylcarbinol inhibit sheep liver esterase 4–5 times more powerfully than the D-forms. With rabbit liver esterase, the inhibition by both D- and L-forms was about the same. The optical antipode specificity of the sheep liver esterase is understandable but the lack of similar selectivity by rabbit liver esterase is not.

The study of the hydrolysis of asymmetric esters by enzymes led Bamann and Laeverenz (1931) to the observation that liver esterase hydrolyzes more rapidly the D-component in a DL-mandelic acid ester composition. When the optically active forms were used separately as substrates, the L-component was more susceptible to the action of the enzyme. The implication of this work is that the D-component in the mixture inhibited the esterase action on the L-isomer and itself underwent hydrolysis.

The relationship between structure of aliphatic alcohols and their inhibi-

tory activity against liver esterase was investigated by Glick and King (1931) who found that the effect of normal primary alcohols increases rapidly as the hydrocarbon chain lengthens. Among the seven isomers of amyl alcohol, the inhibitory effects decrease as the steric hindrance about the hydroxyl group increases. Kernot and Hills (1933) confirmed the essential feature of this work.

As might be expected, certain fatty acids were also inhibitory to the action of liver esterase (Weber and King, 1935). With increasing chain length, the inhibitory power of the salts of the normal fatty acids increased for liver esterase up to sodium laurate and then decreased and finally vanished with palmitic and stearic acid salts. In general, the inhibitory power of the soaps was much less than that of the corresponding alcohols. Unsaturation tended to increase antimetabolite potency. The activity of ortho-substituted benzoic acids suggests a lack of specificity in this system. It may be that any carboxyl substituted unit in which steric hindrance is not a factor would possess a degree of displacer power.

Urease

The enzyme urease shows extreme specificity. It attacks only one compound, urea, producing ammonium carbamate. The sulfhydryl groups make this enzyme susceptible to inactivation by the ions of heavy metals: silver, mercury, copper, cadmium, and lead. Inhibition of this type is nonspecific but represents a manifestation of displacement in the broadest sense.

Few studies have been made of specific inhibition of urease and none has been sufficiently detailed to permit delineation of basic displacement type. Takeuchi (1933) reported inhibitory activity displayed by arginine and histidine. These might therefore be classified as naturally occurring inhibitors. In addition, he found a series of urea derivatives which prevented the action of urease.

Acid ureides were weakly active, and of a series of ureido acids, only ureidoglutaric was strongly inhibitory. Takeuchi also prepared a series of glycyl peptides and reported that glycylaspartic acid was strongly inhibitory.

The hypothetical compound, $\text{NH}_3\cdot\text{CO}(\text{NH}_2)_2$, supposedly formed in slightly alkaline solutions of urea, has been proposed by Deasy (1947) as the inhibitor of urease activity, manifesting itself in experiments designed to show the inhibitory effect of urea on urease.

Certain Enzymes Involved in Carbohydrate Metabolism

Inferentially, the structural similarities of the carbohydrates would point to the existence of natural metabolite analogues; and such analogues do exist.

The initial studies in this field reported the inhibition of glycolysis by glyceraldehyde. Adler et al. (1937) noted the blockage by the aldehyde of

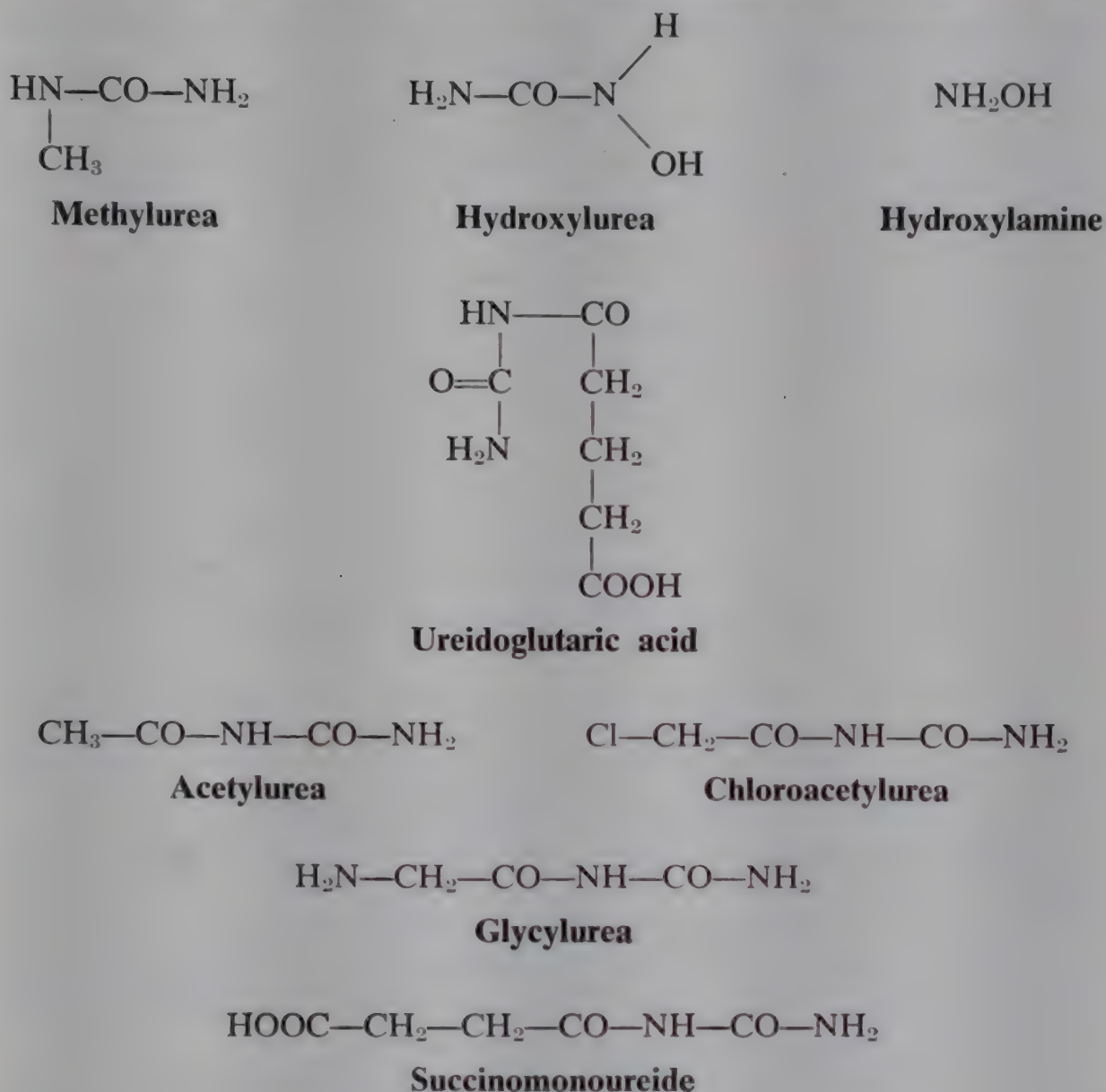


FIG. 2. Inhibitors of urease.

lactic acid formation from glucose or glycogen in cell free brain extract and sarcoma slices. On the other hand, no stoppage of lactic acid synthesis from hexose-monophosphate or diphosphate in these same test systems was noted. Using muscle extract, glyceraldehyde caused a cessation of lactic acid formation from glycogen but not from hexose-monophosphate. Süllmann (1938), using as test system the isolated lens, observed an identical action by glyceraldehyde.

There have been numerous reports of the inhibitory effect of a sugar against the action of an enzyme whose substrate is a similar sugar moiety. In this line, Kuhn (1923) found that yeast saccharase— β -h-fructosidase—was inhibited by fructose and β -glucose, but not by α -glucose. On the other hand, taka saccharase was blocked by α -glucose but not by β -glucose nor fructose. He interpreted these findings as indicating that yeast saccharase was a fructosidase, and taka saccharase a glucosidase. As this is a rather

remarkable instance of substrate blockage the structural formulae of the sugars are given in Fig. 3.

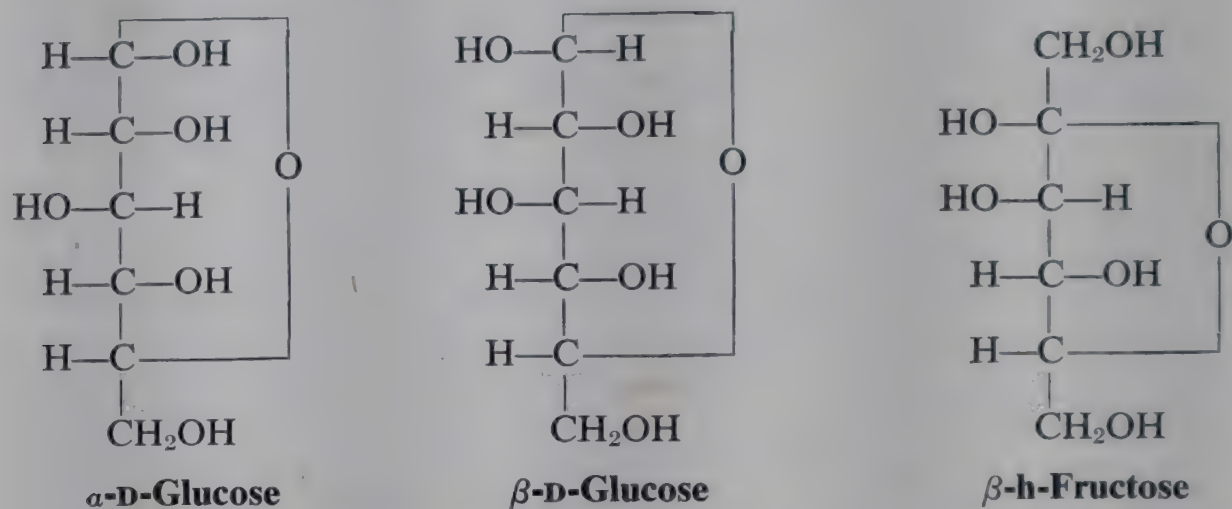


FIG. 3. Substrate enzyme inhibitors of β -h-fructosidase.

Again, in the case of levansucrase, the enzyme forming glucose and levan from sucrose, inhibition is manifested by D-xylose, L-arabinose, D-glucose, L-sorbose, and lactose (Hestrin and Avineri-Shapiro, 1944).

Related blockage phenomena are those of ascorbic acid for the β -amylases of plants (Waldschmidt-Leitz and Purr, 1931–32, 1932), and of saccharic acid for β -glucuronidase (Karunairatnam and Levvy, 1949). In the latter case, the hydrolysis of phenylglucuronide by the glucuronidase was the test system. The action of saccharic acid was competitive and a value of 6×10^{-5} M was obtained for K_i .

There are no corresponding *in vivo* studies although the inhibiting action of mannose upon the growing plant (Wachtel, 1943) and the toxicity of lactose for the rat (Mitchell and Dodge, 1935) and the plant (Knudson, 1916) may be manifestations of competitive inhibition.

Three reports have been made on substrate analogue interference with the action of phosphorylases and transphosphorylases involved in carbohydrate metabolism. Glucose inhibits α -glucosan phosphorylase, which transforms glycogen into glucose-1-phosphate (Cori and Cori, 1940), and sucrose phosphorylase, which catalyzes the formation of glucose-1-phosphate from sucrose (Doudoroff, 1943). Slein (1950) used yeast and brain hexokinase, which is involved in the transfer of phosphate from adenosine triphosphate to hexoses, and hexose pairs such as mannose and glucose. In this system, these sugars act as substrates and inhibitors at the same center of the enzyme. The K_i and the K_s values for each are identical. With brain hexokinase, either glucose or mannose almost completely inhibited the utilization of fructose. Fructose had little effect on the phosphorylation of the aldohexoses.

Miscellaneous

The one item we may consider miscellaneous concerns the depression of respiration of kidney slices by trans-aconitic acid and the reversal thereof by cis-aconitate, citrate, and succinate (Saffran and Prado, 1948).

Recapitulation

The term Substrate brings to mind a multitude of molecules, among them all of the prime and intermediary products of amino acid, fat, and carbohydrate metabolism. It includes all molecules foreign to the body which are subject to "detoxication" reactions, and those which undergo any type of metabolic alteration. Thus, the entire fields of pharmacology and chemotherapy come within limits under the substrate category. Toxicology in its broadest aspects is probably no more than a reflection of substrate displacement and enzymatic inactivation. With this scope of application in mind and considering phenomenal successes with such substrate displacers as fluoroacetate and malonic acid, it is apparent that here we have mirrored the limitations of completed research and the potentialities of future investigation.

Use of the term Absolute in reference to enzymatic specificity represents a manifestation of loose terminology. Absolute enzymatic specificity indicates that a proteolytic enzyme will not act on a polysaccharide or some equivalent limitation and does not imply specificity related to a single molecule. It is probable that only relative specificity exists and this statement is made in view of the extreme specificity of urease-urea relationship. The design of biological systems is entirely relative; in fact, relativity of design in metabolic systems is here proposed as an essential attribute of life itself.

The future development of the concept of differential enzymatic concentrations as the basis of tissue specificity of pharmacological, chemotherapeutic, or toxicological agents will form the very foundation of medical science. This theory should be viewed in its broad aspects because the tissue containing the smallest concentration of a given enzyme and therefore theoretically most dependent upon that enzyme for normal function will be affected equally by an inhibitor designed to prevent the action of the enzyme by direct displacement, or one designed to inhibit cofactor function, block substrate, or interfere with product activity.

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Chapter 2

PHARMACOLOGICAL ASPECTS

1. Pharmacological Agents Altering Enzymatic Mechanisms Associated with the Parasympathetic Nervous System.
 - a. Cholinesterase.
 - b. Choline Acetylase.
 - c. Antispasmodics. Agents Blocking or Modifying the Parasympathomimetic Action of Acetylcholine.
2. Pharmacological Agents Altering Enzymatic Mechanisms Associated with the Sympathetic Nervous System.
 - a. Dopa Decarboxylase.
 - b. Amine Oxidase.
 - c. Phenolases.
 - d. Sulfoesterase.
 - e. Adrenolytics.
3. Pharmacological Agents Altering Enzymatic Mechanisms Associated with Histamine.
 - a. Histidine Decarboxylase.
 - b. Histaminase.
 - c. Antihistamines.
4. Biological Antagonism as It Relates to the Pharmacology of Narcotics and Hypnotics.
 - a. Barbiturates.
 - b. Morphine and Related Molecules.
5. Miscellaneous.
6. Comment.

The distinction between pharmacology and toxicology is in the final analysis quantitative and not qualitative. Chemotherapy can be considered a subdivision of the general science of pharmacology. On the basis of material reviewed in this and subsequent chapters, the author offers the concept of biological relativity as the underlying phenomenon of both these fundamental sciences. This theory of biological relativity will be emphasized throughout and its details integrated in the final chapter.

Biological relativity as applied to the immediate subject brings into one concept many well-known observations, two of which are important here. The first concerns the relativity of enzyme inhibitors, the selectivity of antimetabolites based upon structures bearing similarity to natural substrates, cofactors, etc. which are normally involved in vital enzymatic trans-

formations. The second is that of the relative concentration of susceptible enzymes in various tissues and the assumption that any given inhibitor will act primarily against that tissue containing a minimum of the enzyme. In other words, the pharmacological action of a drug is based upon relativity as applied to enzyme susceptibility and tissue concentrations. The same basic issues apply to chemotherapy, with the addition of the factor of relative parasite and host enzyme concentrations.

Pharmacological Agents Altering Enzymatic Mechanisms Associated with the Parasympathetic Nervous System

It is apparent that the chemical agent responsible for parasympathetic activity is acetylcholine. A correlative to this is that such activity will depend upon the concentration of acetylcholine at a given reactor site at a specific time. It seems probable that rules governing kinetics of enzymatic activity as related to substrate-inhibitor will also apply to neurohormonal compounds such as acetylcholine. The following factors would determine available or effective concentration: rate of formation or addition, rate of destruction or removal, and presence of displacers.

The rate of formation is determined by the reaction kinetics of choline acetylase. The rate of destruction is determined by enzymatic activity of cholinesterase. The relative activity of these two parasympathetic enzymes will determine acetylcholine concentration. The presence of displacers or pharmacological inactivators determines availability of a given quantity of the parasympathomimetic agent. The factor of addition is not within our province as it implies the injection or other mode of administration of the drug. The phase of removal may well come within our province but will not in general be considered in view of indeterminate findings.

Cholinesterase

Cholinesterase and those agents altering its activity will be considered first due to the extensive literature. This enzyme was discovered in 1930 by Engelhart and Loewi and found to possess specific capacity for the hydrolysis of acetylcholine to its components, acetic acid and choline. While Stedman et al. (1933) demonstrated the inactivity of liver esterase on acetylcholine, the duality of cholinesterase was subsequently established by Alles and Hawes (1940) and Glick (1945). The true cholinesterase hydrolyzes only choline esters while the other—pseudocholinesterase or non-specific cholinesterase—hydrolyzes other esters in addition. The specificity of true cholinesterase is questioned by Zeller et al. (1949) who report that this enzyme hydrolyzes such compounds as ethoxyethanol acetate, desoxycorticosterone acetate, ketopropanol acetate, phenyl acetate and even acetylsalicylic acid.

Specific and nonspecific cholinesterases differ in their occurrence, ki-

netics, specificity, and inhibition characteristics. The nonspecific enzyme occurs in blood serum, liver, and in the pancreas. The specific form is found in red blood cells, brain, and nervous tissues. Mendel and Rudney (1943) have reported that brain tissue contains only the true form of cholinesterase. Distribution of cholinesterase in nervous tissue is a point of great importance and the details have been presented by Nachmansohn (1943) who found the enzyme localized at the neuronal surfaces and concentrated to the greatest degree at the synaptic points—ganglionic, central nervous system, motor end-plates.

Acetylcholine formation is directly connected with nerve action, i.e., with the transmission of nerve impulse. The esterase functions in the removal of the parasympathomimetic drug and as its action is essentially irreversible plays no role in resynthesis. Kinetically, the difference between the two enzymes is that the true form acts on more dilute solutions of acetylcholine as contrasted to the pseudo-form. The specificity of these functional nonidentical enzyme twins differs not only in the action of the nonspecific form on noncholine esters but also in that the specific form hydrolyzes acetyl- β -methylcholine but not benzoylcholine; the reverse is true for the nonspecific form (Mendel et al., 1943; Nachmansohn and Rothenberg, 1944).

In consideration of inhibitors of cholinesterase (CE), it must be held in mind, as pointed out by Koelle and Gilman (1949) in their review of anticholinesterase drugs that there is no absolute correlation between inhibitor capacity and pharmacological function by virtue of that activity. A drug acting primarily as an anticholinesterase at one site might directly act on effector cells at another point.

Physostigmine, an alkaloid from the Calabar bean, was the first highly effective inhibitor of CE to be studied (Loewi and Navratil, 1926), and it was subsequently found to be a competitive agent (Eadie, 1942). The kinetics of this antimetabolite effect were reported by Straus and Goldstein (1943) who found that one molecule of physostigmine or one molecule of acetylcholine probably combines with one active center of cholinesterase.

A comparison of the structure of pilocarpine and of physostigmine (Fig. 1) should reveal the distinctive structural characteristics of an agent acting directly with one acting by virtue of cholinesterase inhibition. Pilocarpine acts directly.

Prostigmine, which has been the drug of choice in the treatment of such clinical states as myasthenia gravis, is an effective inhibitor of CE (Hottinger and Bloch, 1943); however, it also possesses a direct action on skeletal muscle. This was demonstrated by Riker and Wescoe (1946) who found that prostigmine evoked contractions of muscle in the presence of diisopropylfluorophosphate (DFP) which is a specific inhibitor of cholinesterase. The action of the prostigmine in this experiment would have to

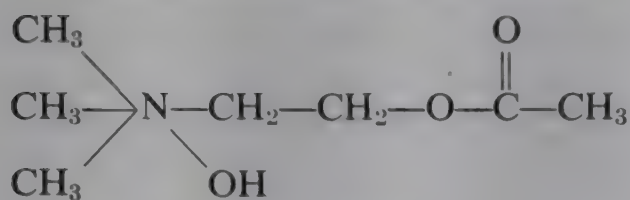
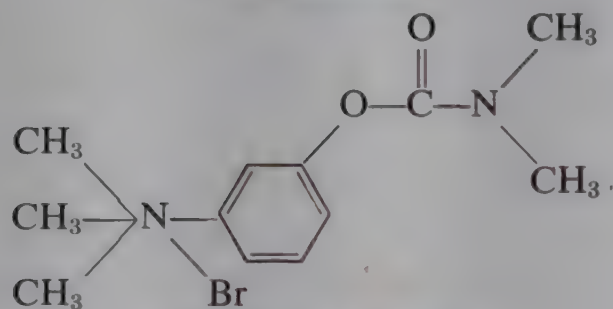
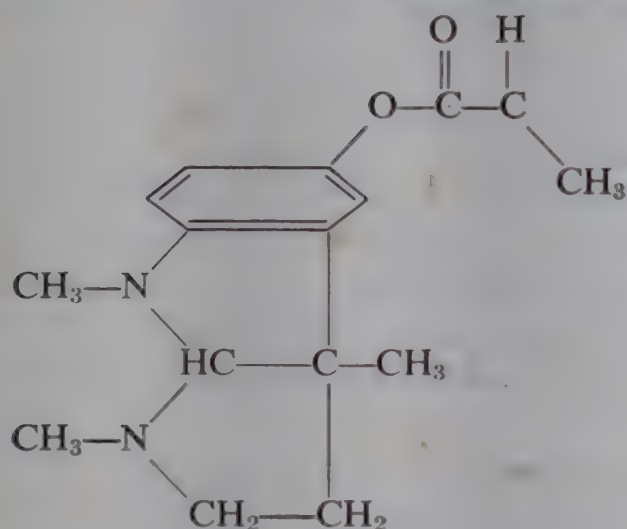
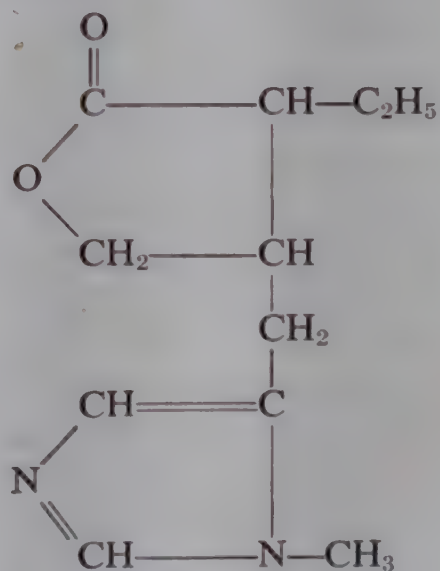
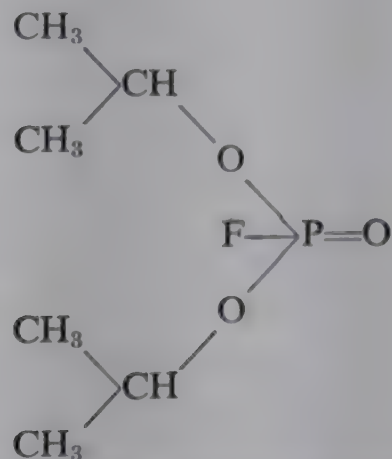
**Acetylcholine****Neostigmine bromide
(prostigmine bromide)****Physostigmine
(eserine)****Pilocarpine****Diisopropylfluorophosphate**

FIG. 1. Chemicals modifying the activity of parasympathetic enzymes.

be direct or at least not motivated via CE because this enzyme was completely inactivated by the DFP. Such experiments do not reflect the quantitative or even the qualitative components of the activity of the drug as used clinically but they do indicate the potentiality of a dual action. Riker and Wescoe interpret their results as indicating the classification of prostigmine with acetylcholine as an agent acting directly on skeletal muscle. This conjecture might hold true under the conditions of their experiment but must be limited thereto until actual evidence accumulates for their broader application.

While it is not indicated that extensive listing be made of all cholinesterase inhibitors, a few are given for orientation. In the class of reversible and noncompetitive inhibitors Goldstein (1948) lists methylene blue, acriflavine, morphine, atropine, strychnine, amphetamine, phenobarbital, sulfanilamide, procaine, choline, and acetyl- β -methylcholine. One of the nitrogen mustards, namely $\beta\beta'$ -dichlorodiethyl-N-methylamine, is a competitive inhibitor (Adams and Thompson, 1948).

Atropine, the most universally used inhibitor of parasympathetic activity, has been reported to inhibit the action of CE in human blood (Schaller, 1942). This is doubtless an example of direct action on a nerve receptor site combined with a relatively minor affinity for the CE activity center. It is to be anticipated that structural similarities will be found on the receptor site of cholinesterase and cell receptor position with resultant overlap in activity. A given molecule may inhibit the enzyme and yet stimulate the cell or it may inhibit both.

Caffeine, a well-known central nervous system stimulant, is a highly specific inhibitor of true CE (Nachmansohn and Schneemann, 1945). Theobromine also possesses this action; quinine on the other hand is more effective against the nonspecific esterase.

Potentialities for the existence of numerous natural inhibitors and stimulators of CE are indicated by the observations of Aron et al. (1944) that glycine, alanine, proline and especially arginine, lysine, and histidine increase the activity of serum cholinesterase, nonspecific form. These investigators stated that the intravenous injection of 0.2 grams of histidine HCl increased serum cholinesterase activity by approximately 50 per cent.

Work such as that of Aron et al. (1944) brings into focus the question of the relative importance of nonspecific cholinesterase as a factor in acetylcholine metabolism. Hawkins and Gunter (1946) offered evidence supporting the contention that the nonspecific form plays no significant part in the in vivo splitting of acetylcholine when they studied the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl) trimethylammonium bromide. This prostigmine analogue inhibited almost completely and selectively the nonspecific form of CE yet following injections of this compound no symptoms suggesting the accumulation of acetylcholine appeared. This is pre-

sumptive evidence only, as factors such as permeability might well cloud the issue.

During the course of chemical warfare research in the period of and following World War II, extensive investigations were made on irreversible CE inhibitors belonging to the series of alkyl phosphates. Bloch (1943) and Hottinger and Bloch (1943) first observed the irreversible inactivation of CE by a phosphate ester, tri-*o*-cresyl phosphate. Later, Adrian et al. (1947) studied diisopropylfluorophosphate (DFP) and demonstrated its activity in inhibiting CE. The irreversibility of this action was discovered by Mackworth and Webb (1948) who were unable to restore enzymatic activity by dialysis.

Similar compounds possessing this CE inactivation characteristic include hexaethyltetraphosphate (HETP) (DuBois and Mangun, 1947), tetraethylpyrophosphate (TEPP), and members of the classes of polyphosphates, phosphophosphines, thiophosphates, and sulfonephosphates (Brauer, 1948). From consideration of a series of 16 phosphate esters, Brauer was able to draw certain conclusions concerning structural attributes necessary for CE inhibition. All active compounds had in common the grouping P—O—R where R is alkyl or aryl. This, however, was not the only criterion. In some pairs of closely related compounds inactivity and activity alternated as follows: triethylphosphate, tetraethylphosphate; trimethylphosphate, dimethylfluorophosphate; tri-*p*-cresyl phosphate, tri-*o*-cresyl phosphate. The active member in each of these pairs has a high free energy content.

The kinetics of action of these phosphate esters have been studied by Brauer (1948) who worked with HETP and TEPP. The relationship between concentration of inhibitor and degree of enzyme inhibition was linear over a wide range, indicating a stoichiometric mechanism of interaction with resultant destruction of both the alkyl phosphate and the enzyme. Physostigmine and similar reversible inhibitors of CE apparently react with the same moiety as the alkyl phosphates. The preceding statement is based upon the observations of Koster (1946) who noted that animals could be protected against DFP by prior administration of certain reversible inhibitors. Koelle (1946) extended study of this phenomenon and by *in vitro* studies demonstrated that physostigmine, neostigmine, and carbaminoylcholine protected CE against DFP.

While DFP inhibits cholinesterase immediately, the irreversible inactivation is a matter of time and other factors (Nachmansohn et al., 1947, 1948). Under proper experimental conditions, a period of a few hours may elapse during which the enzyme-inhibitor complex can be dissociated and the enzyme reactivated. The formation of a loose addition complex followed by a chemical reaction which cannot be reversed is postulated.

Kinetic studies on cholinesterase were first made by Roepke (1937) who

demonstrated that the inhibition of CE by physostigmine or prostigmine is competitive and that equilibrium is reached almost instantaneously. Prior to the work of Easson and Stedman (1936) the reversible inhibition of CE had been regarded entirely as monomolecular and first order but these investigators assumed that the enzyme centers were present in infinitesimally small concentration and that the reaction was of the second order and bimolecular. Straus and Goldstein (1943) extended this concept and established so-called zones of behavior. In the first zone, the enzyme concentration is small and virtually all of the inhibitor exists in the free state. Inhibition is under these conditions a function of inhibitor concentration and the reaction is monomolecular. In the other zones, the enzyme is present in greater concentrations and the reactions between enzyme and inhibitor are bimolecular in type and stoichiometric. As indicated by these investigators, the kinetic type of inhibitor-enzyme reaction would vary in the body depending upon the enzyme concentration.

The dynamics of the inhibition of CE by reversible and irreversible inhibitors have been considered by Augustinsson and Nachmansohn (1949) who found that TEPP inactivated the enzyme irreversibly and immediately while DFP caused the irreversible reaction at a relatively slow pace. Another observation of interest in this report was the shift in optimal substrate concentrations produced by physostigmine and prostigmine.

The action of anticholinesterases should not be regarded as directed solely against the enzyme. Evidence supporting this assertion is varied and ranges from permeability studies to toxicology. The permeability experiments established the acetylcholine-cholinesterase system as a factor in the membrane characteristics of different cells and tissues. Either physostigmine or prostigmine modifies the permeability of erythrocytes (Greig and Holland, 1949), blood-aqueous humor barrier (Swan and Hart, 1940), and rat muscle (Thompson and Tice, 1941). The tendency of anticholinesterases to increase permeability might be said to be the result of CE inhibition, causing the accumulation of acetylcholine.

The capacity of anticholinesterases for the inhibition of brain dehydrogenases has been established by Michaelis et al. (1949) who studied physostigmine, DFP and TEPP. The potency of the alkyl phosphates is indicated by their greater action compared to methylfluoroacetate. Another enzymatic mechanism blocked by anticholinesterases is that of procainesterase. This has been demonstrated in vitro (Kisch, 1943) and in vivo through increased procaine toxicity following use of agents such as physostigmine (Conway et al., 1949).

In addition to the drugs mentioned, the anticholinesterase component (specific or nonspecific) of the activity of many other pharmacological agents doubtless plays a role in their in vivo effects. The list of such agents is extensive and includes: urethanes, geneserine, ephedrine, antipyrine,

choline, priscoline, privine, otrivin, chloral hydrate, barbitol, evipan, phenobarbitol, chloroform, ethyl alcohol, tribromoethanol, phenothiazine, methylene blue, acriflavine, morphine, morphine derivatives, caffeine, theobromine, strychnine, and many others.

Choline Acetylase

The second enzyme primarily associated with the parasympathetic system is choline acetylase. Nachmansohn and Machado (1943) isolated it from brain and from the electric organ of the eel. The enzyme under anaerobic conditions formed acetylcholine in a system consisting of choline chloride, sodium acetate, sodium fluoride, physostigmine, and adenosinetriphosphate. This is an instance in which two enzyme inhibitors were used to demonstrate the existence of a new enzyme. Sodium fluoride inhibited phosphatases and physostigmine blocked the esterase. They found that the enzyme was inhibited by sodium iodoacetate, iodine, and copper. Cyanide was inactive.

Following dialysis, which decreased activity of the enzyme, the addition of L-glutamic acid or cystine restored normal function. Alanine, methionine, and glutamine had some effect. Later, Nachmansohn and his associates (1943) reported that both succinic and citric acids were effective in increasing the activity of dialyzed extracts.

In 1947 Lipmann et al., announced that the coenzyme for acetylation was a pantothenic acid derivative. His group (Lipmann and Kaplan, 1946) had previously demonstrated the essentiality of this cofactor for the acetylation of choline in vitro. Furthermore Riggs and Hegsted (1948) had reported defective acetylation by pantothenic acid deficient animals.

The potentiality that pantothenic acid displacers might prove effective inhibitors of choline acetylase in vivo led several groups to study the problem. Phenylpantothenone failed (Novelli and Lipmann, 1948) as did an entire series of pantothenic acid displacers (Martin et al., 1950). Success crowned the efforts of Cohen et al. (1949, 1949a) who demonstrated the inhibition of the formation of coenzyme A by pantoyltaurine and the inhibition of the action of preformed coenzyme A by D-(2-(pantoylamino)-ethylsulfono)-4-nitroanilide.

While succinic and citric acids activate dialyzed choline acetylase preparation (Nachmansohn et al., 1943), sodium malonate decreases or inhibits this enzyme (Torda and Wolff, 1944). This work carried out by Torda and Wolff also disclosed the effectiveness of fumarate in increasing choline acetylase activity. Sodium oxalate had no effect. The inhibition of the enzyme by α -keto acids such as pyruvic, phenylpyruvic, p-hydroxyphenylpyruvic, and α -ketoglutaric had been noted by Nachmansohn and John (1944, 1945).

The intermediary metabolism involved in acetylcholine formation would

seem to be the formation of acetate from fatty acids, the conversion to an activated form, namely acetyl phosphate, and the transfer of the acetyl radical to choline by an enzymatic mechanism the cofactor of which is a pantothenic acid-containing unit.

Inhibitors fit into this pattern as follows: iodoacetate by inactivation of sulfhydryl groups, malonate by blockage of succinic acid metabolism channels which may lead to acetate, and pantothenic acid displacers by interference with action of coenzyme A.

A new class of choline acetylase inhibitors, the bioflavonoids, must now be considered. Beiler et al. (1950) proceeding from the knowledge of the —SH— grouping of choline acetylase (Nachmansohn and John, 1945) and the susceptibility of that grouping to quinones or quinone forming compounds (Nachmansohn and Berman, 1946) demonstrated the inhibitory action of a series of flavonoids on the enzyme. Morin was the most powerful compound tested, producing a 65 per cent inhibition at a concentration of 0.1 mg. per ml.

Antispasmodics. Agents Blocking or Modifying the Parasympathomimetic Action of Acetylcholine

The mechanism of action of antispasmodics remains vague but there are facts which tend to place all pharmacological activity in the displacement category and the antispasmodics are no exception. Displacement of acetylcholine from a reaction site is theoretically the basis of efficacy, and an attempt will be made to support this contention with fact.

For purposes of limitation, the discussion will be restricted to neurotropic antispasmodics. The action of such agents it will be recalled is determined by interference with the effect of acetylcholine on an isolated strip of intestine. The musculotropic drugs in this group are tested against the spasmodic effect of barium chloride on an intestinal segment. The underlying mechanism is difficult of interpretation.

A further complication in an attempt to study agents blocking acetylcholine is that such chemicals can be divided into three general groups: (1) those effective against the neuromuscular transmission activity of acetylcholine: e.g., curare; (2) those active against the "muscarinic actions" of acetylcholine: e.g., atropine; and (3) those antagonistic toward the ganglionic acetylcholine effect: e.g., tetraethylammonium. While there is cross-over, the degree of specificity manifested by these drugs indicates that there is a fundamental difference in the reaction site for acetylcholine on the several effectors; and conversely that the drugs carry structural modifications causing one to be more suited to displacement of acetylcholine from one effector site than from another.

The action of atropine on various known enzymes might offer an explanation of its activity but absence of any correlation dictates acceptance of

the displacement by this drug of acetylcholine from an effector site. For example, atropine does not interfere with the formation of acetylcholine following nerve stimulation, which is evidence that it does not interfere with choline acetylase (Sollman, 1942). Its action on cholinesterase is inhibitory (Schaller, 1942), which would indicate a parasympathomimetic activity rather than its actual spasmolytic effect.

Clark (1926) first studied the quantitative relationships between acetylcholine and atropine. He reported a constant ratio of metabolite to antagonist over a wide range of concentrations which indicates competition for the same effector.

These facts combined with the work of Moe et al. (1950) in which they demonstrated the competition between atropine and acetylcholine in the spinal cat (using the pressor response of acetylcholine as a criterion of ganglionic activity) conclusively establish the displacement theory as the mechanism of action of this spasmolytic agent.

Atropine is itself subject to hydrolysis by the enzyme, atropinesterase. The concentration of this enzyme is subject to wide species variation. The rabbit can eat belladonna leaves without manifesting toxicity because of the enzymatic degradation of the atropine. The kinetics and properties of this esterase have been studied by Glick (1940).

Tetraethylammonium (TEA) has a specific capacity to produce ganglionic blockade. The action of an entire series of related molecules on isolated frog muscle was studied by Kulz (1923) and Raventos (1937). Compounds of the type $(\text{CH}_3)_4\text{NX}$ to $(\text{CH}_3)_3\text{N}(\text{C}_4\text{H}_9)\text{X}$ possessed a parasympathomimetic effect but as the chain length was increased, the effect decreased and finally with an 8-carbon unit the molecule became antagonistic. $(\text{CH}_3)_3\text{N}(\text{C}_8\text{H}_{17})\text{X}$ and tetraethylammonium completely blocked the action of acetylcholine on the frog heart.

Moe and Freyburger (1950) state "Whatever the intimate nature of the TEA block of ganglia, it is almost certainly competitive." They base this statement on the work of Konzett and Moe (1950) who perfused the isolated cervical ganglion of the cat with TEA and completely prevented response to a dose of acetylcholine which in the absence of the TEA produced a submaximal effect; increase in the amount of acetylcholine caused stimulation again.

Prior to the work of Moe and Freyburger (1950), Clark and Raventos (1937) had studied the antagonistic action of TEA for tetramethylammonium and attributed it to competition for a common receptor because of the similarity of the molecular structure of the two agents. Furthermore, Loewi (1946) proposed displacement by cations as the basis of action of TEA in antagonizing the effect of an inorganic ion, potassium.

Of the three types of neurotropic blocking agents, the atropine type and the tetraethylammonium type have been reviewed and their action associ-

ated with competitive antagonism. This leaves the third type which was the curare or neuromuscular blockage group for consideration. Cowan (1936) found that curarine reverses the acetylcholine depolarization of muscle and concluded that the two drugs act on the end-plate in the neuromuscular junction by mutual antagonism. The correlation of curarine-acetylcholine antagonism and curarine-end-plate potential tends to establish acetylcholine as chemical mediator for the potential. Delicate adjustments of concentrations of curarine-acetylcholine can bring about excitation which is above, below, or just threshold (Kuffler, 1943).

Another member of the curare neuromuscular blockage group is 1,10-bis-trimethylammonium decane (decamethonium salts). This compound was a member of a group of polymethylene bisquaternary ammonium salts studied by Barlow and Ing (1948) and Paton and Zaimis (1949). From the standpoint of competitive systems, the counteraction of the decamethonium compound by the lower homologue of the series 1,5-bis-trimethylammonium pentane is of interest. The neuromuscular paralysis caused by the C-10 compound is not reversed by anticholinesterases but is by the C-5 compound. C-10 is also antagonized by d-tubocurarine chloride (Castillo et al., 1949).

Pharmacological Agents Altering Enzymatic Mechanisms Associated with the Sympathetic Nervous System

Dopa Decarboxylase

Phenylalanine and tyrosine metabolite analogues are considered in detail in Chapter 7, and one phase of that subject is properly dopa decarboxylase. In the interests of continuity, the data presented there in detail will be summarized here.

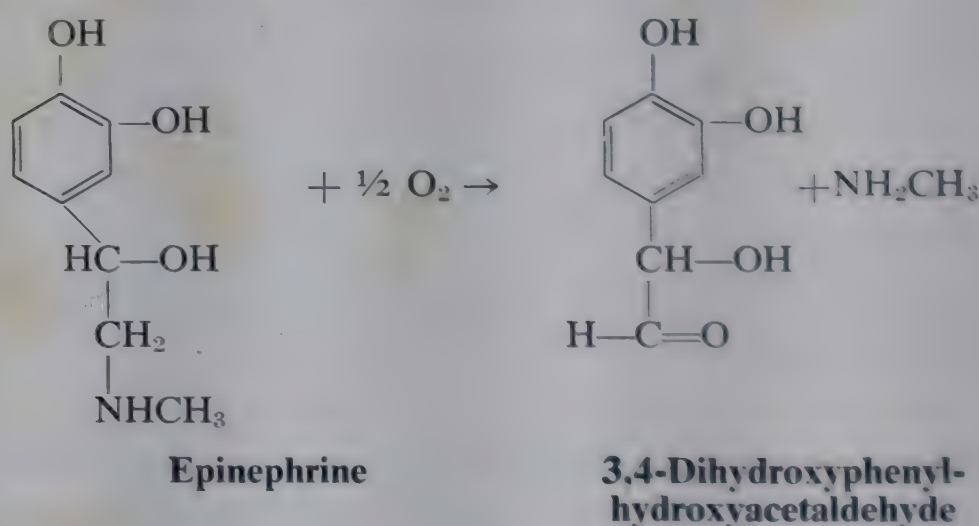
Assuming substrate and enzyme concentrations to be essentially constant, the determining factors in the formation of pressor amines would be enzymatic activity, the balance or relative activity of dopa decarboxylase and other enzymes involved in epinephrine-arterenol formation, and amine oxidase, phenolases, and sulfoesterase which are concerned in pressor amine destruction. Studies of the natural and synthetic inhibitors of dopa decarboxylase are few in number.

In the synthetic group, two approaches have been made; one via cofactor displacement, one by substrate displacement. Folic acid antagonists (see Chapter 12) effectively reduce the activity of the decarboxylase by virtue of structural similarity to the coenzyme (Martin and Beiler, 1947, 1948). Tyrosine and phenylalanine analogues block the action of dopa decarboxylase by substrate displacement (Martin et al., 1950, 1950a). In the natural group, no experimental work has been reported to date unless one considers the studies of Sullivan et al. (1932) as a manifestation of natural

inhibitors of dopa decarboxylase. These workers noted the counteraction of the toxicity of tyrosine by cystine. Glycine was subsequently shown to possess the same activity as cystine (Martin, 1947).

Amine Oxidase

Amine oxidase or more properly monoamine oxidase occurs in liver, kidney, brain, and lungs and possesses the capacity to deaminate tyramine, aliphatic monoamines, and secondary amines of the epinephrine type. In these reactions, the amine is converted to an aldehyde (Blaschko et al., 1937) in accordance with the following formulation:



After a consideration of inhibition studies of monoamine oxidase we shall review the role played by this enzyme in the control of sympathetic activity in the body. The substrate specificity of the enzyme is broad (Hartung, 1946) but it does not act on secondary carbinamines such as 2-aminoheptane, diamines with each of the amino groups attached to a primary carbon, disubstituted amines, and certain other amines.

In the interests of brevity, all inhibitors of monoamine oxidase are listed in Table I.

The kinetics of inhibition of amine oxidase were studied by Ohta (1948) who found that the system was typical of the competitive type. Certain features common to all inhibitors included: a free amino group adjacent to a terminal methyl, optimal activity with a 3-carbon side chain, reduced potency with substitution on the hydrocarbon chain or the amino group, and increased power if the benzene ring was substituted. Ohta also noted that a phenyl or cyclohexyl group at each end of the chain prevented complex formation and that there was no optical specificity.

As an example of inhibitor enzyme affinity, the K_m values for isoamylamine and amphetamine are 8×10^{-4} (Alles and Heegaard, 1943) and 4×10^{-4} (Mann and Quastel, 1940) respectively.

The enzymatic concatenation of amine oxidase activity has been sug-

Table I
INHIBITORS OF AMINE OXIDASE

<i>Compound</i>	<i>Substrate</i>	<i>Action</i>	<i>Reference</i>
Cocaine	Tyramine	+	Bernheim & Bernheim, 1945a
Ephedrine	"	+	"
Caprylic alcohol	"	+	"
Cyanide	"	+	"
Methylene blue	"	+	"
Indole	"	+	"
Indoleacetic acid	"	+	"
Cadaverine	Amylamine, synephrine	—	Blaschko, 1939
Histamine	"	—	"
Ephedrine	Tyramine	+	Blaschko, 1938
Phenylisopropylamines ..	"	+	"
β -Phenylisopropylamine ..	"	+	Blaschko, 1940
d-Desoxyephedrine	"	+	Orzechowski, 1941
Cocaine	Sympathin, epinephrine	+	Torda, 1943
Veritol	Epinephrine	+	Heim, 1947
Ephedrine			
Pervitin			
Enzymatic oxidative products of catechol and epinephrine	"	+	Friedenwald & Herrmann, 1942
Secondary & tertiary carbinamines	"	+	Heegaard & Alles, 1943
Urea	Tyramine	+, Irreversible	Bhagvat et al., 1939
Caffeine and other purine derivatives	Epinephrine	+	Ohta, 1945
Amphetamine derivatives	Tyramine	+	Ohta, 1948
Coniine	Epinephrine	+	Werle and Pechmann, 1949
Piperidine			
Nicotine			
Secondary carbinamines	"	+	Pugh & Quastel, 1937; Frontjes, 1942
Secondary amylamine ..	"	+	Heegaard & Alles, 1943
Monoamidines	"	+	Blaschko and Duthie, 1945, 1945a
Alkyl diamidines			
Aromatic amidines			
Diguanidines	"	+	"
Diisothioureas			
Local anesthetics	"	+	Philpot, 1940

gested by Govier et al. (1946) as a coupled reaction between a dehydrogenase (amine oxidase) with pressor amine as substrate and lactic dehydrogenase in the presence of pyruvate. The cytochrome-cytochrome oxidase system was also involved. The proposal was based on the finding that the oxidation of tyramine by rat liver in vitro was stimulated by cytochrome C, coenzyme I, α -keto acids, methylene blue, and aluminum and calcium ions.

Among the amidines, diamidines, diguanidines and diisothioureas tested as amine oxidase inhibitors, Blaschko and Duthie (1945a) studied a series of alkyl diamidines and found that their affinity for the enzyme increased to a maximum with the 12-carbon chain unit. There was a parallel among the related alkyl diamine substrates in that enzyme affinity was maximal at the 12-carbon unit, indicating that both natural substrate and inhibitor attained optimal structural characteristics with the same chain length.

Blaschko (1940) was among the first to associate specific inhibition of amine oxidase with pharmacological activity in vivo. He correlated the awakening characteristics of a series of inhibitors and obtained positive results. From his findings, he concluded that in general inhibitors possess

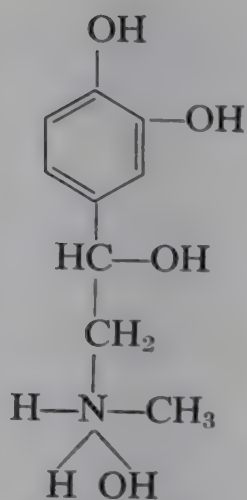
the structure
$$\begin{array}{c} | \\ \text{R}-\text{C}-\text{CH}_2-\text{NH}-\text{CH}_3 \\ | \end{array}$$
 Similar generalizations on the

structure of inhibitors of amine oxidase were made by Heegaard and Alles (1943) who proposed that secondary and tertiary carbinamines were apt to be antagonists. The molecular size of the alkyl and phenylalkyl secondary carbinamines played a role as α -methyldiamylamine and α -methylphenylpropylamine were the most active in their respective series.

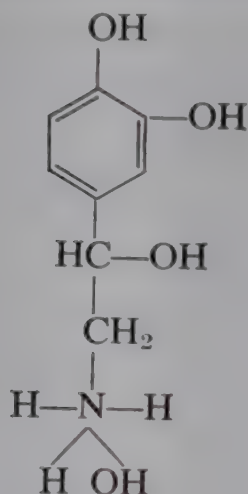
Support for the contention of a correlation between biological activity of sympathomimetic compounds and their enzymatic inactivation was offered by Beyer (1941) who stated that phenylpropylamines having the amino group on the terminal carbon atom of the side chain were oxidized in the presence of amine oxidase. If the compound had one or two hydroxyl groups on the benzene ring it was oxidized by phenolase. Finally, where the amino group was on the carbon atom adjacent to the terminal one and the molecule contained no hydroxyls on the ring, neither enzyme could use it as substrate.

Again, Ohta (1946) who had studied the inhibition of amine oxidase by some 32 phenylalkylamines concluded that the necessary condition of inhibition was that the amines must have a methyl group at the end of the alkyl chain and that there exists a parallel relation between inhibiting action and effect on the central nervous system.

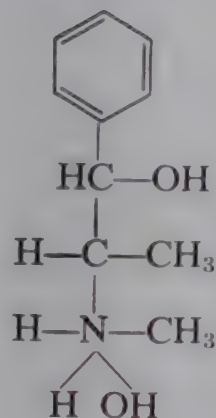
The work of Brun (1947) is interpreted by him as failing to support the hypothesis that ephedrine interferes with amine oxidase. His negative conclusion was based upon vasoconstrictor action of ephedrine when applied directly to arteries of muscle, omentum, and mesentery of the white rat



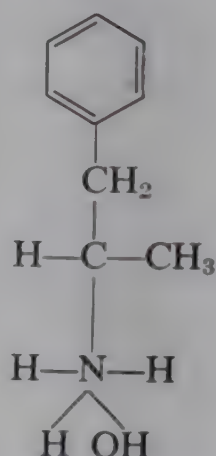
Epinephrine



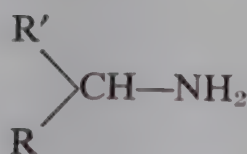
Arterenol



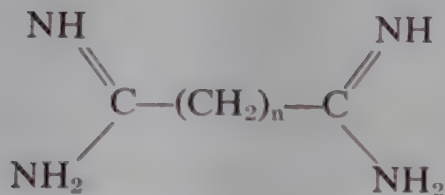
Ephedrine



Amphetamine



Secondary carbinamines



Alkylene diamidines

FIG. 3. Chemicals modifying the activity of enzymes or effector cells of the sympathetic nervous system.

immersed in Ringer's solution. Epinephrine possessed a similar action which in the arteries of the abdominal muscles was suppressed by ephedrine. Direct action of ephedrine is not established by observations on intact animals; moreover the suppression of epinephrine action by ephedrine might be interpreted as blockade of sympathetic effector cell reaction sites.

A similar interpretation can be placed on the work of Ahlquist (1946) who demonstrated the reversal of the action of β -phenyl-n-propylmethylaniline by β -(p-hydroxyphenyl)-isopropylmethylaniline. While Ahlquist does not himself agree, it is felt that effector cell site blockage of the sympathetic system with consequent unmasking of parasympathomimetic action offers the explanation.

Bacq (1949) has assembled the case against the physiological involvement of amine oxidase in the removal of epinephrine from the circulating blood. We shall review each of these and make alternate proposals.

Bacq's first objection is that amine oxidase is concentrated in liver, intestine, and central nervous system and is absent or present only in low

concentrations in tissue known to inactivate epinephrine (Richter and Tinney, 1939). To use this as a valid point against physiological action of amine oxidase is to assume that no other channel of epinephrine inactivation exists, and this is not borne out by the facts.

Another point we will attempt to refute is based upon the relation of time involved in amine oxidase action and the extreme dilution of epinephrine in blood (Kohn, 1937). This assumes identical *in vitro* and *in vivo* activity of enzymatic mechanisms, and Philpot (1940) has indicated that there is no correlation. Further, the action of amine oxidase might be against some far more susceptible amine precursor of epinephrine.

Still another argument used by Bacq (1949) to invalidate the theory of the physiological importance of amine oxidase is that the eviscerated animal is not more sensitive to epinephrine despite the high concentrations of the enzyme in the liver (Markowitz and Mann, 1929). This hardly seems valid in view of the profound physiological and biochemical readjustments which would follow such drastic surgery.

The contention that the almost identical quantitative destruction of epinephrine by liver and nonpregnant cat's uterus (Bacq, 1936) mitigates the importance of amine oxidase in the physiological state, can be refuted on the basis of the lack of knowledge of the role played by other inactivating mechanisms. The dominant role might be played by amine oxidase in the liver, for example, and by phenolase in the uterus.

At this point in his presentation, Bacq (1949) attacks directly the concept of Gaddum and Kwiatkowski (1938, 1939) that ephedrine sensitizes to epinephrine by virtue of blockage of amine oxidase. The strongest issue raised is that ephedrine contracts the chronically denervated nictitating membrane of the anesthetized and adrenalectomized cat, which means that epinephrine would not be present (Bacq, 1937). Refutation here follows the line that pressor amines of many types are known and that most of them would be susceptible to the action of amine oxidase; any one of these might be protected against amine oxidase by ephedrine. It is not logical to assume that ephedrine protects epinephrine alone, since Blaschko (1938) has already proved its protective action for tyramine when this amine forms the substrate for amine oxidase.

Each line of evidence proposed by Bacq (1949) is subject to question. Consider one last point, namely, that efficient inhibitors of amine oxidase such as ethylurethane do not sensitize *in vivo* to epinephrine (Blaschko et al., 1937a). *In vitro* actions quite generally do not parallel *in vivo* effects. *In vivo*, destructive mechanisms may remove ethylurethane or there may be natural substrates tending to block or reverse the action it would otherwise have.

While the matter of the relative importance of the enzymatic activity of amine oxidase for the pharmacological effect of epinephrine remains un-

settled, the power of metabolite analogues to modify the kinetics of this organic catalyst is established beyond question.

Phenolases

Epinephrine can be destroyed by oxidation of the hydroxyl groups with the formation of adrenochrome. The reaction which is associated with phenolases unquestionably occurs *in vitro*, but its *in vivo* significance is obscure. One point to be considered is that the phenolase is involved in the formation as well as the destruction of epinephrine and therefore arguments indicating lack of functional significance of phenolases in such destructive reaction do not in any sense eliminate possible importance of the enzyme in controlling pressor amine concentration at a given site.

Chapter 7 deals with tyrosine and phenylalanine metabolism, but a few points from that section should be offered here in the interests of continuity of the presentation of the metabolism of the sympathetic biochemical system. The conversion of phenylalanine to epinephrine has been demonstrated by an isotope technic. The initial phases of this conversion involve tyrosinase (a phenolase), converting phenylalanine to tyrosine and hence to dihydroxyphenylalanine, which is then decarboxylated enzymatically. Dopa decarboxylase cannot utilize either phenylalanine or tyrosine as substrate; tyrosine decarboxylase does not occur in the mammalian system. It is obvious therefore that phenolase activity will control substrate concentration for the action of dopa decarboxylase, which in turn supplies the needed chemicals for epinephrine formation.

The inhibition of tyrosinase can be caused by β -2-thienylalanine, various fluorinated phenylalanine and tyrosine derivatives, N-acetyltyrosine, hydroquinone, aromatic amines, and aromatic aminobenzoic acids (for detail, see Chapter 7).

The same inhibitors of the *in vitro* oxidative destruction of epinephrine by tyrosinase are effective in a test system involving dopa-kidney tissue (Martin et al., 1942). Ephedrine has an inhibitory effect on the oxidation of epinephrine by tyrosinase, a fact of interest in view of the relation of this compound to amine oxidase. It is apparent that the same inhibitors of tyrosine oxidase generally act against any of the normal substrate transformations, including those of phenylalanine, tyrosine, dihydroxyphenylalanine, or epinephrine.

The importance of phenolase activity in epinephrine oxidation in the body seems slight viewed in the light of current data. After Neuberg (1908) described the phenolase which inactivates epinephrine, determinations were made of its concentration in tissues, the resultant values being so low the assumption was made that the enzyme could play no role in the biological inactivation of the pressor amine (Bhagvat and Richter, 1938). Later, it was proposed that those tissues inhibited by epinephrine contain an enzy-

matic concatenation with specificity and that in this instance phenolase type enzymes are of great importance (Bacq, 1938).

Whereas adrenochrome is pharmacologically inactive, it is interesting to note that adrenoxine (Heirman, 1937, 1938) which is formed by the action of a phenolase, is a strong depressor. It is possible that adrenoxine is a metabolic product of epinephrine, so closely related structurally as to displace the epinephrine molecule, preventing its action and producing a drop in blood pressure. While adrenoxine has not been isolated in pure form, its pharmacological potency is of sufficient magnitude to suggest the importance of future research along these lines. As a mydriatic, adrenoxine is 10,000 times more powerful than epinephrine (Heirman and Goffart, 1939).

While adrenochrome is pharmacologically inactive, it is not biochemically so. Green and Richter (1937) found that it played a role as a hydrogen carrier, and that the concentrations present in vivo rendered probable its function in this capacity in normal physiology. Much of the action of adrenochrome is probably due to a property it possesses in common with other quinones: namely, that of the reversible oxidation of the sulfhydryl group. The action of this epinephrine oxidation product as a "vitamin P" substance is probably based on its SH oxidizing effect (Derouaux, 1941). Similarly, it inhibits enzymes of the glycolytic cycle; viz., hexokinase, phosphohexokinase (Wajzer, 1946, Meyerhof and Randall, 1948).

Bacq (1949) closes his review of the subject by stating "an important fraction (of epinephrine) is simultaneously oxidized to indole substance." Considering the reviewed reports, this leads to the conclusion that Beyer (1941) properly classified sympathomimetic compounds in groups subject to the action of phenolases or amine oxidase. The compounds subject to the action of phenolase were those having one or two hydroxyl groups on the benzene ring.

p-Aminobenzoic acid inhibits the action of tyrosinase on epinephrine (Martin et al., 1942) and if such phenomena were produced in vivo, the administration of p-aminobenzoic acid should cause certain pharmacological manifestations paralleling those of the sympathomimetic drug itself. p-Aminobenzoic acid in doses of 1 and 2 grams per kilogram does produce a mild hyperglycemia in dogs (Ansbacher et al., 1944). Similarly, this aromatic amino acid causes a rise in blood pressure in the anesthetized cat, reversed by 2-1-piperidylmethyl-1,4-benzodioxane (Martin and Ichniowski, 1944). Both observations substantiate the contention that phenolases are operative in vivo in epinephrine destruction, and that inhibitors of phenolase activity prevent epinephrine metabolism and have as a result an indirect sympathomimetic effect.

Another important mechanism of inactivation of sympathomimetic agents, is that of esterification of the phenolic groups.

Sulfoesterase

Bacq (1949) subscribes to the theory that esterification is the major pathway of epinephrine inactivation in the body. Richter in 1940 was the

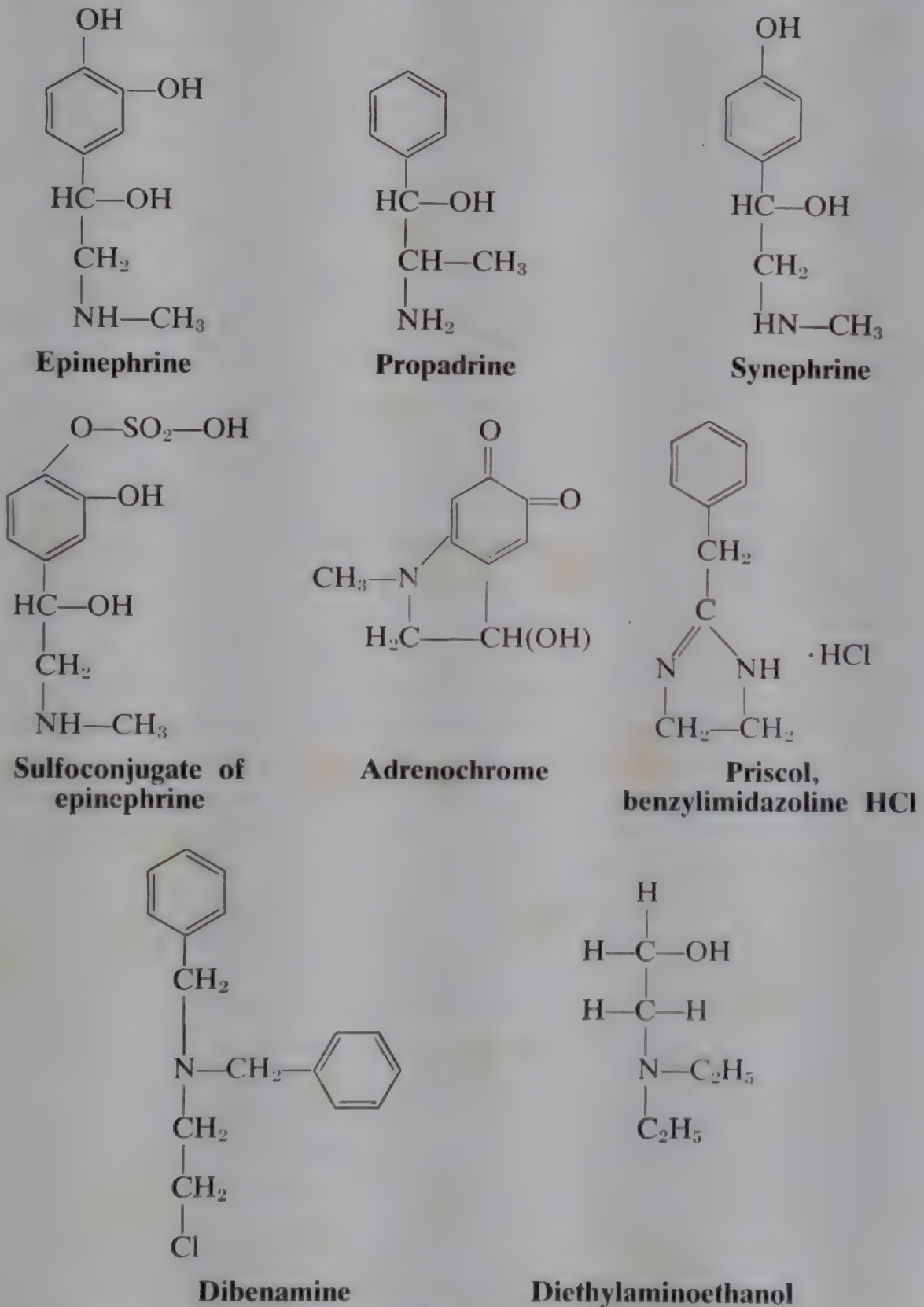


FIG. 4. Compounds modifying the involvement of phenolases and sulfoesterases in the biochemistry of the sympathetic nervous system.

first to present evidence in support of this metabolic channel. He ingested large amounts of both isomeric forms of epinephrine and isolated from the urine a compound which had the characteristics of a sulfoconjugate. The molecule on hydrolysis yielded epinephrine. Tests for glucuronic acid were negative; those for sulfuric esters positive.

Sympathomimetic amines such as cobefrine, which are not oxidized by amine oxidase, are esterified to a greater extent than those subject to the action of the oxidase, e.g., epinephrine (Beyer and Shapiro, 1945). It is probable that there are three and possibly more types of sympathomimetic amines; those inactivated mainly by monoamine oxidase, those destroyed primarily by phenolases, and those modified to the greatest extent by sulfoesterase.

Torda (1943a) found that cocaine inhibited the ester formation of phenols, a fact suggesting that part of the potentiating action of cocaine may be through its inhibition of the esterification of the phenolic epinephrine molecule. The sensitization produced by many phenols to epinephrine and to sympathetic stimulation (Bacq, 1936a) has been interpreted by Richter (1940) as due to a competition by these phenols for the sulfoconjugation-inactivation system. The augmentation of epinephrine action is due therefore to antagonism for a "detoxication" system. That this is a unique instance of metabolite displacement is so not because of the infrequency of such reactions but because it is one of the first of its type to be studied. "Detoxication" reactions are frequently of the variety in which conjugation with glucuronic acid, ethereal sulfate, glycine, cysteine and rarely glutamine, occurs. In general, the conjugation results in the production of a chemical with physiological or pharmacological properties distinct from those of the unconjugated form. Blockade of any of these metabolic sequences would naturally materially alter the biochemical significance of the foreign molecule under consideration.

Bacq (1936a) raises an objection to the concept promulgated by Richter (1940). Resorcinol, which is conjugated to form an ethereal sulfate as readily as catechol or pyrogallol, does not sensitize to epinephrine even if present in concentrations far exceeding that at which catechol is active. While this is a factor for resolution, one interpretation might be that resorcinol does compete for the sulfoesterase reaction site but that this effect is obscured by simultaneous function as a metabolite displacer from a sympathetic system effector site. Another interpretation would be that the compound in vivo potentiates the action of phenolases upon the epinephrine.

Adrenolytics

In accordance with the concept that effector cell reaction sites are similar to those on enzyme surfaces, it is logical to assume that there will be a

large number of molecules capable of displacing "trigger" molecules from reaction sites. In the case of the sympathetic nervous system, there are a host of such compounds. Nickerson (1949) has recently reviewed the field. Our problem concerns the possible integration of these drugs into the sphere of metabolite displacement.

One major difficulty in a consideration of this field lies in the apparent variation in structure of the effector cell reaction site. Stimulation of the sympathetic nervous system elicits certain responses and in no single instance are all of these blocked by any one adrenolytic. Dibenamine, for instance, a typical member of the β -chlorethylamine series of adrenolytics, has no effect upon the excitatory response of the heart to epinephrine (Hunt, 1949). The receptors of the heart apparently differ from those of muscle effectors where dibenamine does exert an antagonistic effect against epinephrine.

Another confusing aspect is the interaction of drugs in the general category of sympatholytics. While such interaction was to have been anticipated by virtue of structural similarities, the confusion lies in interpretation of results. As an example, piperidinomethyl-3-benzodioxane (933F) prevents dibenamine from blocking the vasopressor effect of epinephrine, a fact interpreted by Seed and McKay (1949) as due either to a chemically mediated destruction of dibenamine by 933F or to the fact that the benzodioxane derivative protects the epinephrine receptors from the dibenamine. This may be an instance in which a weaker displacer interferes with the action of a stronger. Another example is the effect of a sympathetic depressor compound such as the isopropyl homologue of epinephrine, which converts to a pressor effect the depressor response to epinephrine induced by an adrenolytic drug (Coret and van Dyke, 1949). Moreover, the depressor sympathetic compound does not produce its customary effect, a phenomenon designated by its discoverers as tapenolysis. Coret and van Dyke offer in explanation of their observations the hypothesis that vascular smooth muscle has sympathetic reaction sites which are excitatory and inhibitory. Sympathetic drugs can react predominantly with either of these or equally with both. Epinephrine reacts with both, isopropylepinephrine with the inhibitory, and norepinephrine with the excitatory. The adrenolytic drug blocks most of the excitatory sites but none of the inhibitory. Following an adrenolytic, therefore, epinephrine can only react with the inhibitory reaction sites. When isopropylepinephrine is added in excess, it combines with the inhibitory sites in a loose manner, and in the experiment the excitatory loci are partially but not completely blocked by the adrenolytic; this leaves some excitatory sites for reaction with epinephrine, which therefore exerts its customary pressor effect.

Another phase to be considered in studying the sympathetic and sympatholytic drugs as metabolite antagonists is the overlap of the sympatholytic

agents into other systems. Typical of this overlap is the cholinesterase inhibitory capacity of β -chlorinated amines such as dibenamine (Bain, 1950) and the partial antihistaminic potency of other β -chloroethylamines (Loew and Micetich, 1948; 1949).

Finally, the observation by Wells and Rall (1948) indicating inhibition of the pressor action of 2-(1-naphthylmethyl) imidazoline HCl by N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine HBr mirrors the complexity of the interacting forces at play in the relative effects of sympathomimetic and sympatholytic agents.

Parallel experiments demonstrating antagonistic effects of structurally related molecules were conducted by Modern and Thienes (1936) who found that propadrine and d-synephrine blocked the action of epinephrine on smooth muscle, whereas d-metasynephrine did not. The earliest report of inhibition of one sympathomimetic agent for another was that of Abderhalden and Slavu in 1909 when they observed that d-epinephrine protected mice against the l-isomer.

A detailed listing of reported instances of competitive blockading mechanisms is not within the scope of this volume but reference is made to other observations on the antagonisms of one sympathomimetic amine for another (Hambourger and Jamieson, 1936; Malorny and Orzechowski, 1940) and to the adrenergic power of some phenylethylenediamines and phenylhydrazines (Benoit and Bovet, 1942; Bovet et al., 1942).

Nickerson and Goodman (1947) proposed that dibenamine and similar compounds function by irreversibly inactivating a hypothetical enzyme system essential for excitatory responses of effector cells to epinephrine or other sympathomimetic agents.

Later, this same group (Nickerson and Nomaguchi, 1948) substantiated their contention by demonstrating that dibenamine does not prevent penetration of sympathomimetic amines to their site of action nor does it paralyze the contractile mechanism of smooth muscle cells. Elimination of these two possible loci of action leaves that of specific combination by the compound at the site of normal effect of a sympathomimetic agent on the cell. The possibility would remain that competitive antagonism may occur during the establishment of the irreversible blockage.

Piperidinomethyl-3-benzodioxane and ergotamine manifest competitive antagonism for epinephrine over a wide range of concentrations (Abdon and Hammarskjold, 1940-41; Gaddum, 1926; Mendez, 1928).

A review of the chemical and pharmacological characteristics of the entire series of compounds functioning as sympatholytics strongly indicates that all types— β -chloroethylamines, ergot alkaloids, imidazolines, benzodioxanes, yohimbine, phenoxyethylamines and others—act by the same mechanism associated with the displacement of the amine-alcohol configuration of epinephrine from a receptor of a sympathetic system effector

cell. With some adrenolytics the reaction is reversible and truly competitive; with others, such as the β -chloroethylamines, it is irreversible and probably associated with chemical combination with the same amine-alcohol receptor. The sympatholytic effects of diethylaminoethanol fit well into this pattern (Rosenberg et al., 1949; Freis et al., 1949).

The most promising clinical application of the adrenolytic or sympatholytic agents lies in the field of hypertension. Nickerson (1950) in his general review concludes that the present use in neurogenic hypertension is effective. While currently this limits the field to hypertension associated with pheochromocytoma and intracranial lesions, it is Nickerson's opinion that future developments will find the adrenolytics used in prophylaxis as well as therapy. He favors the β -haloalkylamines over the dihydro ergot alkaloids and the imidazolines.

In conclusion, the chemical basis of the adrenergic blockage by dibenamine and similar agents, emphasized by Nickerson and Gump (1949), merely places these drugs in the category of irreversible noncompetitive displacers (fluorophosphates, the mercurials, and similar chemicals) and in no sense eliminates them from consideration as biological antagonists.

Pharmacological Agents Altering Enzymatic Mechanisms Associated with Histamine

Histidine Decarboxylase

Histidine is an essential amino acid by virtue of the inability of the animal organism to synthesize the imidazole nucleus. This constitutes proof of the origin of histamine from the nitrilite, as no other imidazole source is available. Chapter 9 reviews the literature on histidine and its metabolite analogues but we are here considering the enzyme, histidine decarboxylase, which decarboxylates the amino acid and forms histamine. The activity of this enzyme will therefore be one of the controlling factors in the concentration of histamine at a given site.

Werle (1936) first described the formation of histamine from histidine by tissue slices. The enzyme, histidine decarboxylase, was later found in extracts of rabbit and pig kidney and in liver. The results of Werle (1936) were confirmed by Holtz and Heise (1937). Optical specificity is shown by the enzyme; it attacks L-histidine but not the D-isomer.

Studies of the inhibition of histidine decarboxylase are few. Werle (1942) observed the antagonistic action of dihydroxyphenylalanine, epinephrine, and similar compounds. Subsequently, studies were made by Beiler et al. (1949) in which they observed the effects of vitamin antagonists, phenolic compounds and agents structurally related to histidine. None of the vitamin antagonists proved effective. In fact, methylfolic acid and pyridoxamine, metabolite antagonists of folic acid and thiamine respectively, actually

stimulated enzymatic activity. This finding was interpreted as due to the presence in the tissue of enzymes which attacked histidine in some other manner. Blocking these enzymes would prevent destruction of histidine and increase substrate concentration for histidine decarboxylase action. Werle (1942) had previously reported that thiamine inhibited the action of histidine decarboxylase.

Phenolic compounds were effective. The extent of inhibition caused by these compounds was dependent on the number of hydroxyl groups present and activity was restricted to phenolic hydroxyl since inositol, a compound rich in nonphenolic hydroxyls, was completely ineffective.

Of the compounds tested, β -2-thienylalanine bore the greatest structural similarity to histidine and it was an effective antagonist in concentrations of 1.0 mg. per ml.

Several flavonoid compounds inhibit the action of histidine decarboxylase *in vitro* and also potentiate the action of ascorbic acid as an antagonist (Martin et al., 1949). The effect of the flavonoids was tentatively associated with their ability to form quinones and react with sulfhydryl groups as proposed by Bartlett (1948). Of the series tested *in vitro*, d-catechin was by far the most powerful, producing a 40 per cent inhibition in a concentration of 0.01 mg. per ml.

If this enzymatic inhibition occurred *in vivo*, d-catechin should prevent the formation of histamine or at least reduce it and so eliminate or modify anaphylactic shock. d-Catechin injected intraperitoneally at levels of 5 mg. per kilogram of guinea pig does in fact stop anaphylactic shock (Moss et al., 1950). In this same study, it was found that catechin had no significant capacity to prevent the pharmacological action of histamine.

Histaminase

Diamine oxidase which constitutes one mechanism for the destruction of histamine was discovered in 1929 by Best. Zeller (1942) demonstrated the lack of specificity of the enzyme, listing as substrates: ethylenediamine, trimethylenediamine, putrescine, cadaverine, spermidine, homologues of spermidine, spermine, agmatine, and histamine.

Zeller (1938, 1938a, 1940, 1941, 1942) has extensively studied the inhibition of diamine oxidase. The competitive antagonism of arginine and spermine with histamine for a reaction site on the oxidase was first reported by him (Zeller, 1938a). In that same year, he (Zeller, 1938) observed the displacement capacity of guanidine and its derivatives in this system. The inhibitory power of the diguanidines increased with the degree of methylation, decamethylenediguanidine being the most powerful. Here, again, the antagonism was of the competitive type. Then in 1940 Zeller et al., added ephedrine and curare to the list.

The most recent study of diamine oxidase is that of Werle and Pechmann

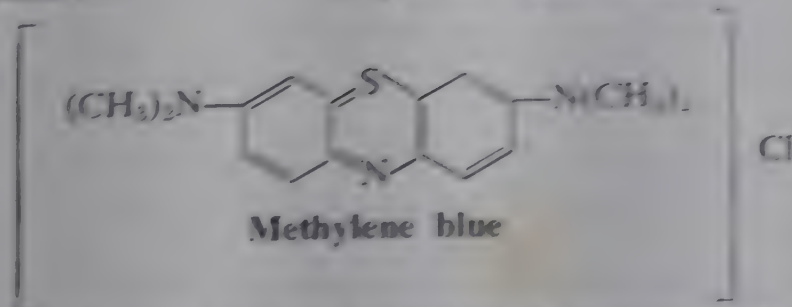
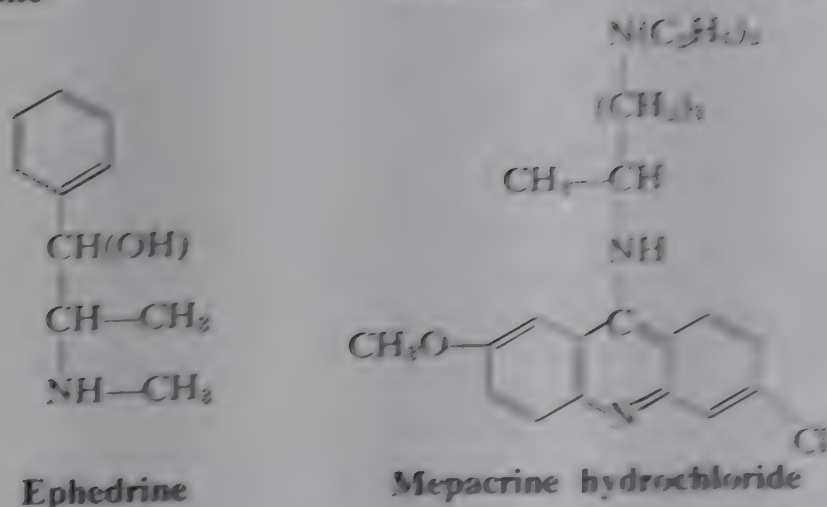
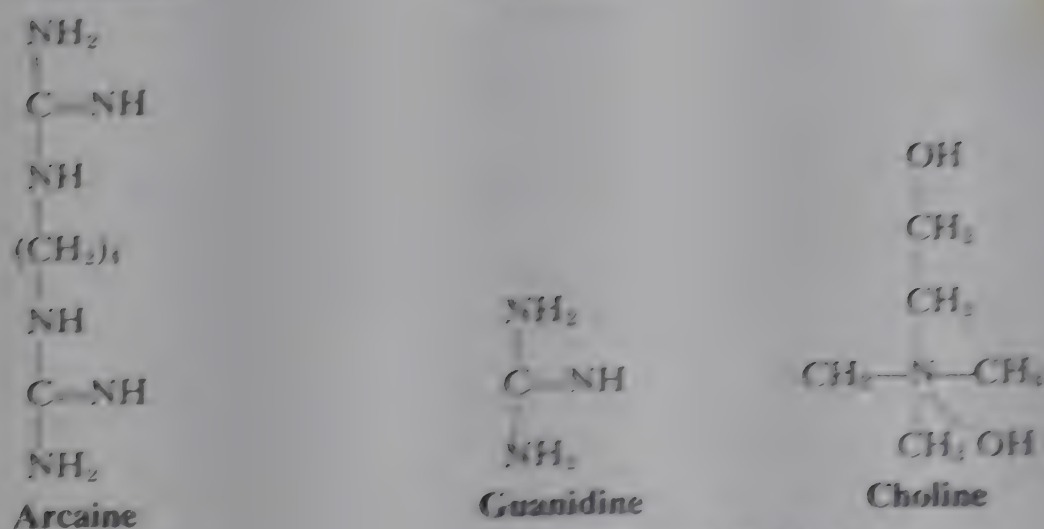
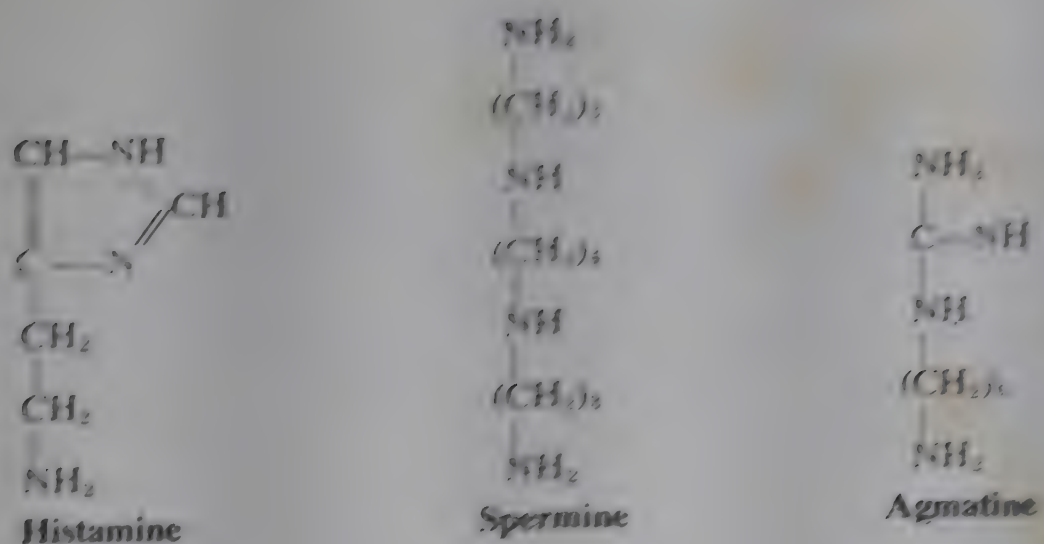


FIG. 5. Substrates for and inhibitors of diamine oxidase.

Table II
INHIBITORS OF DIAMINE OXIDASE

<i>Compound</i>	<i>Substrate</i>	<i>Inhibitory Effect</i>	<i>Reference</i>
Spermine	Histamine and other diamines	+	Zeller, 1938
Arcaine	"	+	" "
Cyanide	"	+	" "
Semicarbazide	"	+	" "
Monoamines	"	+	Zeller, 1942
Methylamine			
Amylamine			
Choline	"	+	Zeller, 1940
Ephedrine	"	+	Zeller et al., 1940
Guanidine	"	+	Blaschko, 1939
Imidazole	"	+	Zeller, 1941
Methyl- and dimethylguanidine ...	"	+	Zeller, 1938
Diguanidines	"	+	" "
Methylene blue	"	+	McHenry & Gavin, 1935
Pyocyanin, Toluene blue	"	+	Zeller, 1938
Thiamin	"	+	Sullman & Birkhauser, 1939; Edlbacher & Becker, 1940
Mepacrine hydrochloride	Cadaverine	+	Blaschko & Duthie, 1945b
Aromatic amines	Cadaverine or histamine	+	Werle & Pechmann, 1949
p-Aminomethyl-phenylsulfonamide ...	"	+	" " "
Caffeine	"	+	" " "
2-Phenylbenzylamino-methylimidazoline	"	+	" " "
Dimethylaminoethyl-benzylaniline	"	+	" " "
Prostigmine	"	+	Keeser, 1939
Potassium salts	"	+	" "
Ascorbic acid	"	+	" "
Ergotamine	"	+	" "
Atropine	"	—, Stimulates	" "
L-Histidine	"	+	Satake & Sibata, 1940
Amino acids	"	+	" "

(1949) who confirming the inhibitory activity of aliphatic monoamines (Zeller, 1942) found that the capacity decreased from primary to quaternary compounds. Aromatic amines proved to be weak displacers. A point of great interest reported in this same paper is the competitive inhibition caused by the antihistaminics such as 2-phenylbenzylaminomethylimidazole and dimethylaminoethylbenzylaniline.

Natural inhibitors of histaminase are found among the amino acids with histidine being the most specific (Satake and Sibata, 1940). Inhibitors are therefore known which may be classified in the natural and synthetic class. This being true the question immediately arises as to the biological significance of diamine oxidase.

Zeller (1942) reviews the physiological role of diamine oxidase. In the mammalian organism, histamine is formed by the action of histidine decarboxylase within the body and by bacterial action in the gastrointestinal tract. Diamine oxidase is largely responsible for the destruction of this powerful depressor agent. Histamine is not the only diamine formed; in certain states (toxemia of pregnancy, parathyroidectomy in dogs, cystinuria) both cadaverine and putrescine are found in the urine. Zeller feels that the high concentration of diamine oxidase in the gut wall is for the destruction of diamines formed by bacterial action in the gut.

Furthermore, Zeller contends that diamine oxidase is coupled with the processes of reproduction, particularly in that spermine, which occurs in sperm, is a substrate for the oxidase. In addition, toxemia of pregnancy which Zeller associates with histamine formation may be due to a failure in the activity of the destructive oxidase. One very interesting interrelationship is brought out by Zeller. Choline blocks diamine oxidase and is a product of the action of cholinesterase; agmatine and cadaverine which are substrates for diamine oxidase block cholinesterase. This is probably another manifestation of biological relativity.

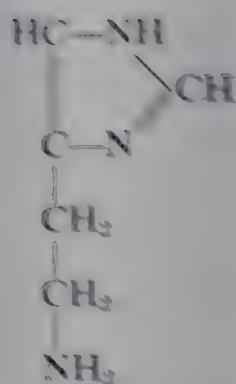
It is safe to state that those conditions generally associated with excessive histamine formation such as shock, perennial hayfever, etc. will be modified in their severity by proper activity of diamine oxidase.

Antihistamines

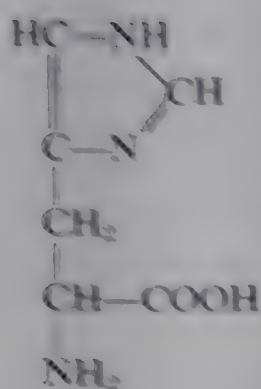
There are three general groups of histamine antagonisms; pharmacological, natural, and synthetic. In the first category are placed those drugs which elicit pharmacological effects, opposed to those of histamine such as the vasoconstrictor substances.

It is more difficult to place drugs such as atropine which neutralize the contracting action of histamine on muscle (Hill and Martin, 1932), in any given classification.

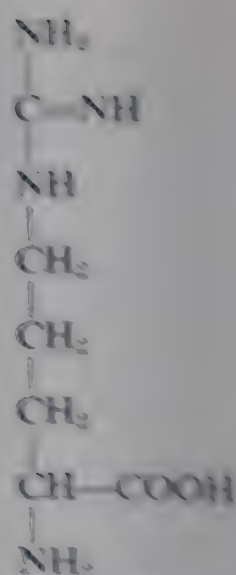
A number of natural antagonists are known. For example, many amino acids inhibit the action of histamine-induced contraction of the guinea pig



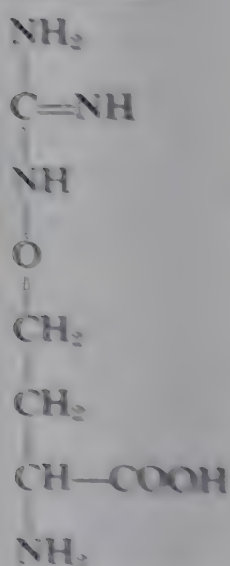
Histamine



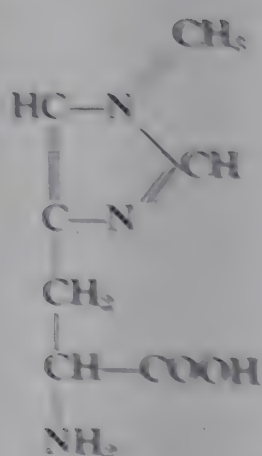
Histidine



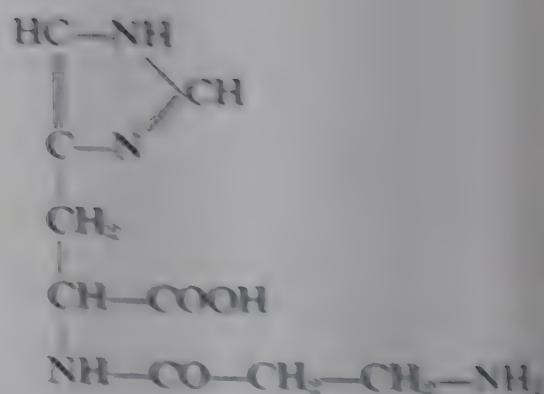
Arginine



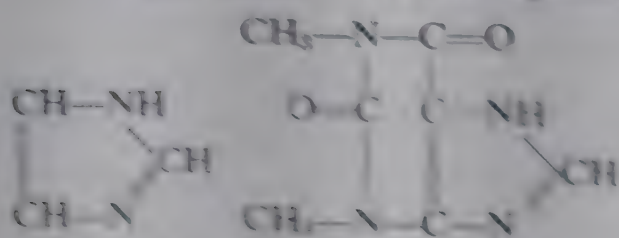
Canavanine



1(3)-Methylhistidine

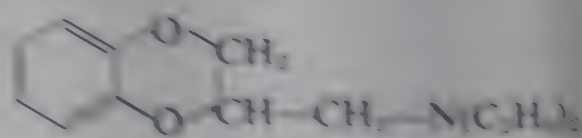


Carnosine

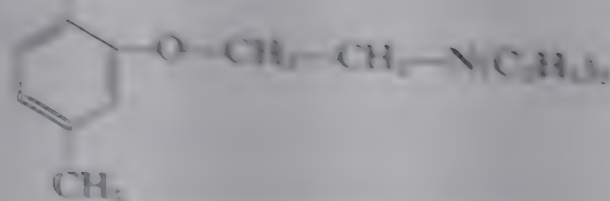


Imidazole

Theophylline



2-Diethylaminomethyl-1,4-benzodioxane



Thymoxyethyldiethylamine

FIG. 6. (See facing page for legend.)

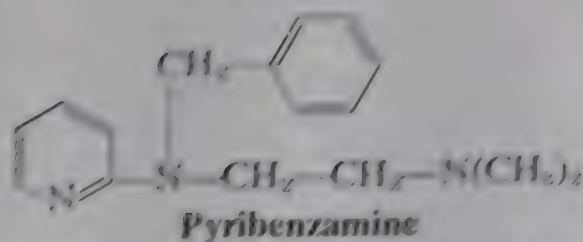


FIG. 6—(Continued). Antagonists of histamine.

intestine; included among these are histidine, arginine, and cysteine (Edlbacher, Jucker and Baur, 1937). Proline, ornithine, and leucine have some activity. The antagonistic effect manifested by these amino acids was reversible in all cases. Confirmation of the work of Edlbacher et al. (1937) was offered by Credner and Schumrick (1943), Sibata (1940), Mackay (1938), and Halpern (1939).

Ackermann and Wasmuth (1939), using the same test system, reported that in addition to the foregoing amino acids, carnosine, certain guanidine derivatives, and polyamines inhibited the action of histamine. The conclusion was reached by Ackermann and Wasmuth (1939) and Ackermann (1939) that the $=NH$ group in the guanidines and secondary amines exhibited competitive antagonism on a mass action basis with the ring $=NH$ of histamine for a reaction site on a receptor cell. Using another test system, the guinea pig uterus, Jadaaohn et al. (1943) observed antagonism of histamine by arcaine, spermine, spermidine, arginine, and triethylenetetramine. They felt that the effect was nonspecific as these substances also antagonized the effects of Pitocin, choline, and acetylcholine on the uterus.

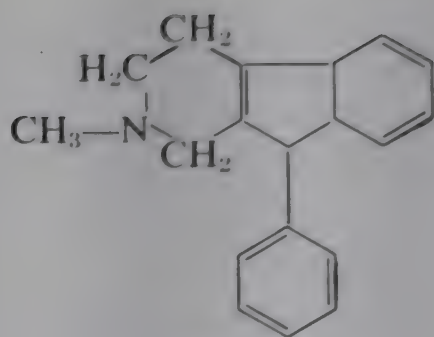
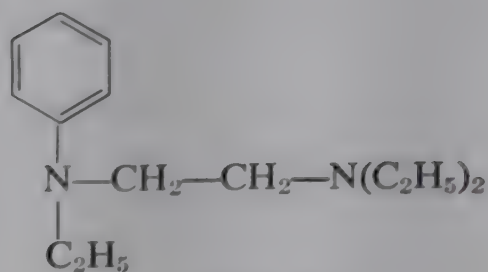
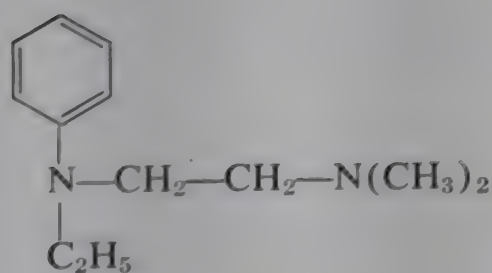
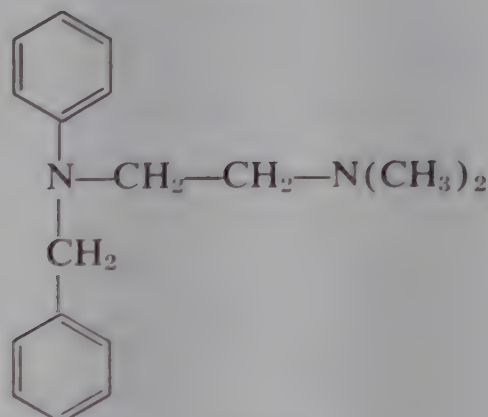
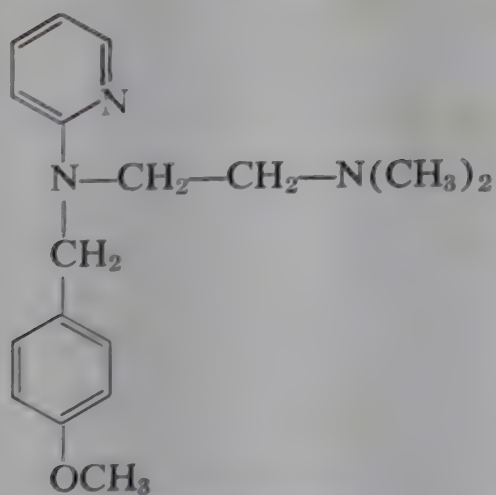
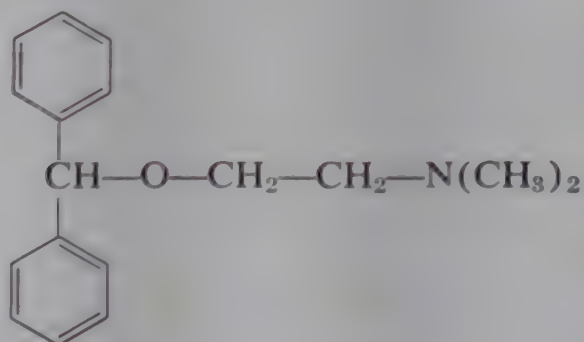
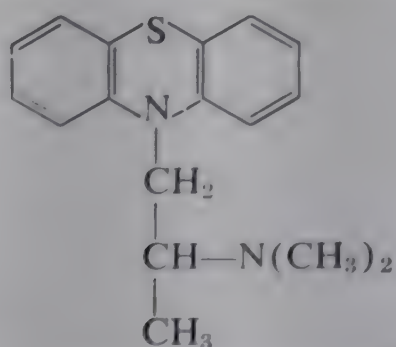
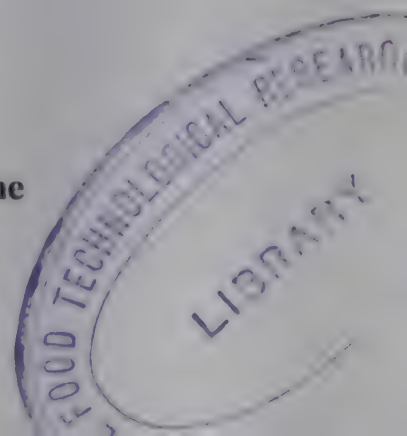
Daily injections of 1 cc. of 4 per cent histidine monohydrochloride for 28 days does not affect the blood pressure but it does markedly diminish the sensitivity of the vascular system of the cat to histamine (Edholm, 1942). The same desensitization is seen using the uterus of the cat as test system. Later, Landau and Gay (1944) studied the problem. First they corroborated the activity of arginine and histidine as antagonists for histamine, using the intestinal strip technic. Then they used strips from the ileum of a sensitized guinea pig and found that the effect of the antigen was blocked by either of the two amino acids. Finally they demonstrated that pretreatment of guinea pigs prevented histamine deaths when the depressor base was given intracardially. A parallel experiment attempting to demonstrate protective action of arginine against anaphylactic shock failed.

Rocha e Silva (1944) studied the effect of compounds derived from histamine, which he considered as models for bound histamine. To this series, he added compounds of histidine and arginine and tested all compounds on isolated guinea pig gut. Benzoyl-L-arginine, benzoyl-L-arginineamide HCl, arginine HCl, histidine HCl, acetyldehydrophenylalanylhistamine HCl, and acetyl-DL-phenylalanylhistamine HCl were all active. Benzoyl-L-arginineamide was many times more active than arginine. The

Table III

ANTAGONISTS OF HISTAMINE

<i>Compound</i>	<i>Test System</i>	<i>Activity</i>	<i>Reference</i>
Vasoconstrictors	Intestine strips	+	Hill and Martin, 1932
Atropine	"	+	" "
Arginine	"	+	Edlbacher, Jucker and Baur, 1937
Histidine	"	+	" "
Cysteine	"	+	" "
Guanidine	"	+	" "
Carnosine	"	+	Ackermann & Wasmuth, 1939
Arcaïne	"	+	"
Agmatine	"	+	"
γ -Guanidobutyric acid . . .	"	+	"
Canavanine	"	+	"
Spermidine	"	+	"
Spermine	"	+	"
Creatine	"	—	"
Creatinine	"	—	"
asym-Dimethylguanidine .	"	—	"
1(3)-Methylhistidine	"	—	"
Anserine	"	—	"
Triethylenetetramine	Guinea pig uterus	+	Jadassohn et al., 1943
Benzoyl-l-arginine	Guinea pig intestine	+	Rocha e Silva, 1944
Benzoyl-l-arginine- amide	"	+	"
Acetyldehydrophenyl- alanylhistamine	"	+	"
Acetyl-DL-phenyl- alanylhistamine	"	+	"
Imidazole	"	+	Morris & Dragstedt, 1945
Theobromine	Bronchi of sensi- tized guinea pigs	+	Emmelin et al., 1941
Theophylline	"	+	"
Caffeine	"	+	"
N,N-Diethyl-N'-phenyl- N'-ethyl ethylenediamine	In vivo	+	Bovet & Staub, 1937
Thymoxyethyldiethylamine	"	+	"
Piperidinomethyl- benzodioxane	Guinea pig intestine	+	Ungar et al., 1937
Thephorin	In vivo	+	Lehmann, 1947
Antergan	"	+	Halpern, 1942
Neo-antergan	"	+	Bovet et al., 1944
Benadryl	"	+	Loew et al., 1945
10-(2-Dimethylamino- isopropyl)-phenothiazine	"	+	Vallery-Radot et al., 1947

**Thephorin****1571 F****2325 R.P.****Antergan****Neo-antergan****Benadryl** **β -dimethylaminoethylbenzhydrl ether****10-(2-Dimethylaminoisopropyl)-phenothiazine****FIG. 7. Antagonists of histamine.**

histamine derivatives showed a potency of the order of magnitude of histidine HCl and benzoyl-L-arginineamide. Rocha e Silva concludes that his results agree perfectly with those of Ackermann (1939) and establish the view that the inhibitory effect of arginine and histidine depends upon a competition between the imino group of these amino acids and the like group of histamine for its reaction site on the cellular receptor.

Before turning entirely to the synthetic metabolite antagonists of histamine, we must consider briefly those molecules structurally related to the depressor amine and possessing similar pharmacological activity. Lee and Jones (1949) investigated a series of compounds with a β -aminoethyl side chain attached to an aromatic, heterocyclic, nitrogen-containing nucleus. There was no correlation between the basicity of the two nitrogen centers of the compounds and their activity. They did reach the conclusion that for histamine activity either the structure $-\text{N}=\text{C}-\text{CH}_2-\text{CH}_2-\text{NH}_2$ or $=\text{N}-\text{C}-\text{CH}_2-\text{CH}_2-\text{NH}_2$ must be in the molecule. One or the other of these structures was present in some 11 different molecules, all showing histamine activity. It is unfortunate that the 12 or more structural analogues which did not manifest histamine potency were not tried as antihistamines. Closely related molecules such as β -phenylethylamine possess a pharmacological activity which is diametrically opposed to that of histamine.

The simplest synthetic which is an effective antihistamine is imidazole (Morris and Dragstedt, 1945), which at levels of 128 mg./kg. reduces by 60 per cent the action of the depressor amine.

The methylated purines comprise another group of molecules effective as antihistamines. Theophylline, theobromine, and caffeine were active in preventing anaphylactic reaction in the bronchi of sensitized guinea pigs (Emmelin, Kahlson, and Lindström, 1941). Theophylline monoethanolamine counteracts histamine, using smooth muscles as test systems. Young and Gilbert (1941) and Gilbert and Goldman (1940) found essentially the same protective action with theophylline ethylenediamine. In their test system, however, the sodium salt of theophylline and the free acid were not effective as antihistamines.

The first synthetic preparations which might be designated as true antihistamines were prepared in Paris at the Fourneau Institute and subsequently reported by Boveri and Staub (1937) and Staub (1939). The most active compounds were N,N-diethyl-N'-phenyl-N'-ethyl-ethylenediamine and thymoxyethyldiethylamine. The total number of compounds studied included 17 analogous amines and 21 ethers. These agents given in doses of 10–20 mg./kg. protected guinea pigs against 2–6 otherwise lethal doses of histamine and against anaphylactic shock. While these were the first of the true antihistamines, Ungar, Parrot, and Boveri (1937) in the same year found piperidinomethylbenzodioxane effective in blocking histamine on the

isolated intestine of the guinea pig, and shortly thereafter Vallery-Radot et al. (1942) discovered the antagonistic power of diethylaminomethylbenzodioxane for histamine.

During 1940 the Rhone-Poulenc Laboratories of France extensively investigated antihistamines and finally settled on Antergan which is N,N-dimethyl-N'-phenyl-N'-benzylethylenediamine as the molecule with the best therapeutic index. The organization marketed the compound in the same year.

In recent years, many new antihistamines have been discovered and marketed, including Pyribenzamine, Thenylene, Diatrin, Chlorothene, Antistine, etc. All are modifications of the basic structures discovered by the French, excepting Thephorin (Lehmann, 1947) which represents a departure from the customary pattern and possesses useful properties for clinical application.

While a review of the field of synthetic antihistaminics is not within the province of this volume, some abstracts from this voluminous literature may facilitate orientation in the field. Halpern (1942) compared the power and specificity of a large series of histaminolytic agents. He found that Antergan was 15 times more potent than 2325 RP and 150 times stronger than 1571 F. The compound 1571 F differs from 2325 RP only in the replacement of a diethyl moiety by a dimethyl, and yet the potency is stepped up by a factor of ten. Antergan differs from 2325 RP only in the substitution of a benzyl for an ethyl radical. For additional structure-potency comparison, Neo-antergan, which is the most powerful drug of this type with specificity (Bovet et al., 1944), compares with Antergan except in the replacement of a benzene by a pyridine unit and by the substitution of a methoxy group on the benzene ring.

The histaminolytic agents possess in varying degree the characteristics of local anesthetics, antispasmodics, sympathomimetics, and sympatholytics. Rosenthal and Minard (1939) first demonstrated the local anesthetic potency of antihistamines using thymoxyethyldiethylamine. As an example of the antispasmodic power (antiacetylcholine action) of some antihistamines, 10-(2-dimethylaminoisopropyl)-phenothiazine suppresses the spasmogenic effect of acetyl- β -methylcholine using the bronchioles of patients as test system (Herxheimer, 1949). Similar studies of the antiacetylcholine power of histaminolytics have been made by Dews and Graham (1946) and Sherrod et al. (1947). Thephorin, according to Lehmann et al. (1947), possesses not only histaminolytic but also sympatholytic properties. This emphasis on overlap in pharmacological activity is made to underline the probable structural similarity of receptor sites for histamine, acetylcholine, and epinephrine. The neurohormonal mechanisms, like all other biological systems, possess only relative, not absolute specificity.

There is, however, no direct correlation between sympatholytic power

and any other pharmacological activity of these drugs. Furthermore, these agents do not achieve their effect by eliciting pharmacological actions opposed to those of histamine. Again, they do not enhance the activity of histaminase nor do they chemically combine with histamine (Staub, 1939). There is no interference with antibody production (Mayer et al., 1946) but there may be blockage of antigen-antibody reactions (Vallery-Radot et al., 1947a).

Attempts to delineate the mechanism of action of Benadryl led Wells et al. (1945) to conclude that direct competition between histamine and the antagonist for a given site of action or receptive substance occurred. The phenomenon was considered as one of displacement of histamine from its reaction site. Furthermore, the antagonism was competitive in nature. The technique used by these investigators involved the determination of blood pressure drop in the absence of Benadryl, and then duplication of that drop in the presence of varying amounts of the antihistamine. The necessity for the utilization of such an involved technique lies in the fact that the dose-effect curve of histamine is not linear. The difference between the two doses producing identical blood pressure fall was in their studies considered to be a true reflection of the amount of histamine blocked. With the values determined experimentally they plotted the effective concentration of Benadryl against the per cent inhibition of histamine and got a curve which was compatible with the Langmuir adsorption isotherm.

The conclusions of Wells et al. (1945) were placed on a firm basis by the concurrence of Halpern and Maurie (1946), Gaddum (1948), and Gilman (1948). One phenomenon associated with antihistamines is their inability to interfere with the normal secretion of gastric hydrochloric acid or with its increased production after the injection of histamine (Sangster et al., 1946). Furthermore, they do not modify materially the toxic effects resulting from the administration of trypsin (Wells et al., 1946). When one considers the probable similarity of the pharmacological overlap receptor sites, it is not beyond logic to expect that receptor points of effector cells in one pharmacological system such as the histamine, will vary. This is offered as the probable explanation of the failure of histaminolytic agents to block gastric secretion.

Another strong point in favor of the displacement theory of histaminolytic action comes from the work of Pellerat (1945, 1947), who determined concentrations of histamine and antihistamine in the blood of patients under treatment. He found that after ingestion or injection of Antergan or Neo-antergan the blood histamine concentration was elevated. As a specific example, he reported an increase in blood levels from 90 micrograms of histamine in total blood and 30 micrograms in the plasma of one patient to a total blood level of 335 micrograms and a plasma level of 154 micrograms one day after the administration of 0.5 grams of the drug. The same in-

creases were noted following the use of several other typical antihistaminic agents. Pellerat concluded that the histaminolytic had displaced the histamine from the tissue receptor sites and that this histamine appeared in the blood.

Pellerat (1945, 1947) also offered a most ingenious proposal in explanation of the so-called "histaminoid" reactions following the use of antihistamines. He suggests as an example that the skin of a patient with urticaria might well be more susceptible and fix the antihistamine preferentially to histamine. Free blood histamine would increase and while there would be some histaminolytic present in the blood, another tissue such as the bronchioles might be more receptive to histamine than to the antagonist, with the result that an asthmatic reaction would be precipitated. This asthmatic reaction would correspond to the so-called "histaminoid" phenomenon.

One of the more recent experiments which establishes metabolite displacement more firmly as the basis of histaminolytic action is that of Barany and Nordqvist (1949). They studied the effect of histamine on the nerve block caused by procaine and also by antihistaminics and found that a nerve block caused by a histaminolytic or by a typical local anesthetic such as procaine could be removed by washing with a solution containing histamine. Furthermore, if histamine were present with the blocking agent, no block resulted even when the concentration of blocking agent was quite sufficient to produce block if applied alone. While it is felt that this technic establishes the reversing capacity of histamine for a histaminolytic nerve block, and that this indicates competitive antagonism, it is not permissible with the evidence at hand to arrive at conclusions relative to the mechanism of reversal of procaine. Certainly, all histaminolytic agents are local anesthetics and if local anesthetics of the procaine type are reversed by histamine, there is a probability that the action of local anesthetics can in general be correlated with their antihistaminic power as determined by a nerve block technic such as that employed by Barany and Nordqvist (1949).

Still another recent and novel study (Sterzl and Krecek, 1949) involves the use of bacteria as test systems for histaminolytics. The bacteriostatic effect of the antihistamines was reversed by histamine or thiamine. The work was interpreted as indicating competitive metabolite displacement of two growth factors, thiamine and histamine. If this work is confirmed, it will be most interesting that thiamine, the antineuritic vitamin, is associated with histamine metabolism in bacteria.

Biological Antagonism as It Relates to the Pharmacology of Narcotics and Hypnotics

The line of demarcation or the point of distinction between a sedative, a hypnotic, and a narcotic vanishes on close scrutiny and it is to be antici-

pated that the basic phenomena of antagonism elicited in one system parallel those of the others. The prime point is that of relative selectivity for the central nervous system.

There are four major categories of theories for narcosis:

1. Those based upon the concept of specific solubility of the anesthetic drugs in cellular lipoids.
2. Those emphasizing alteration in physical phenomena, such as electrical polarity of cerebral cells.
3. Those associating changes in cell metabolism with narcosis.
4. Those proposing modification in cellular oxidative processes.

While the present discussion will concern categories 3 and 4, we do not imply that the others have no merit; rather that biological antagonism would more logically manifest itself in alterations of cellular metabolism and perhaps specifically oxidative processes.

Barbiturates

Barbiturates have become broadly used as sedatives and hypnotics. They are effective and in general side effects are minimal. Numerous modifications of the basic structural unit are known and these vary in dosage, duration of action, and aftereffects. The duration of action is probably a reflection of the speed of metabolism of these agents and may doubtless be profoundly affected by biological antagonism.

Quastel and Wheatley (1932, 1934, 1938) were the first to attempt to explain the action of narcotics as being a reflection of their effect on brain respiration. Using brain slices, they demonstrated a reversible inhibition exerted by barbiturates and other agents on brain respiration. The reversibility was established by washing the slices in a phosphate-glucose-saline medium. The inhibition occurred to the extent of 70 per cent and the reversal was complete. The oxidation of glucose, lactate, and pyruvate was blocked to a greater degree than that of succinate or glutamate.

The proposal of Quastel and Wheatley associating depression of brain metabolism with the action of narcotics was challenged by Fuhrman and Field (1943) who contended that during anesthesia the concentration of narcotic agents in the brain is less than that required for the inhibition of glucose oxidation in vitro. Such results would have to be carefully checked and extended in view of work demonstrating the concentration of diethylbarbituric acid in the diencephalon following intravenous injection into the rabbit (Keeser and Keeser, 1935). Fuhrman and Field found that the power of barbiturates to inhibit rat brain oxidation varied with the 5-position substituents. With increasing length of alkyl side chain in compounds carrying an ethyl or an allyl radical on the other 5-position, the inhibitory capacity increased.

Zorn et al. (1939) used rat liver slices as substrate and found that there

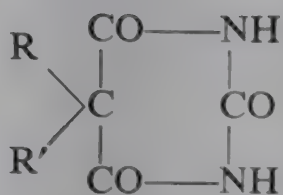
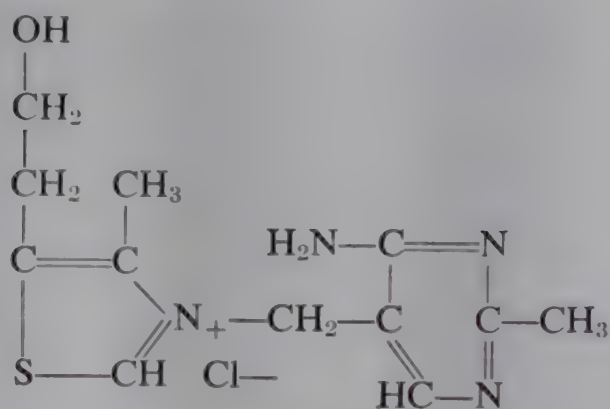
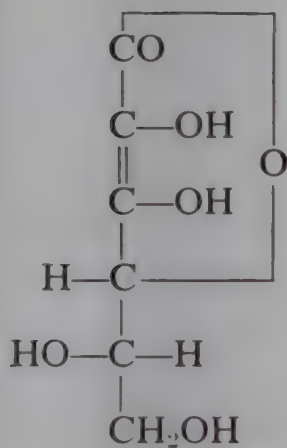
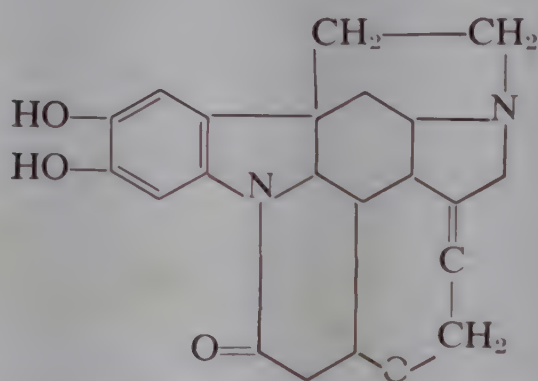
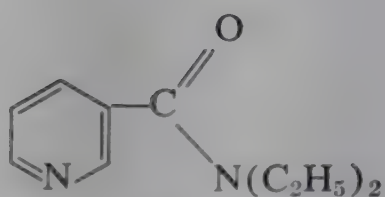
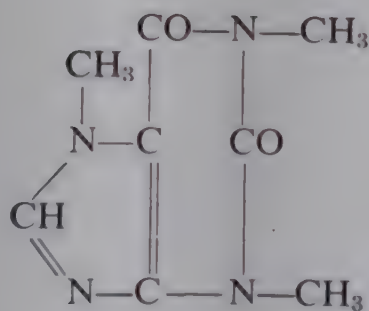
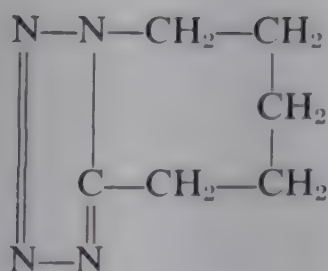
**Barbiturate****Thiamine****Ascorbic acid****Strychnine****Coramine****Caffeine****Metrazol**

FIG. 8. Molecular species studied in relation to pharmacology of barbiturates.

was a reduction in the uptake of oxygen following the addition of barbiturates. They listed the compounds tried in the order of their effectiveness as follows: Amytal, pentobarbital sodium, Neonal, Ortal sodium, phenobarbital sodium, Evipal, Alurate, and Dial. The anaerobic reduction of methylene blue by the liver slices was also inhibited. This tends to show that the selectivity for brain tissue is only relative.

In the final analysis *in vivo* determination would have to be made to ascertain the effect of barbiturates on brain oxidation, and by an ingenious method Wilkins et al. (1949) have accomplished this. Using a dog brain biopsy technic, they found that 0.04 per cent sodium pentobarbital depressed oxygen consumption of dog cortex and that concentrations of 0.08 and 0.12 per cent completely suppressed oxygen uptake.

Attempting to ascertain the basic mechanism of barbiturate action, Eiler and McEwen (1949) discovered the capacity of pentobarbital to inhibit the aerobic phosphorylation reactions of brain homogenates. The drug reduced the formation of high energy phosphate bonds only to the extent of blocking the utilization of oxygen. These effects were observed when the substrate was pyruvic acid but not when succinic acid was present. The fact that the oxidation of succinate is not blocked by barbiturates had previously been announced by Jowett and Quastel (1937). The relation of these observations to the use of succinate as an analeptic in barbiturate poisoning is clear.

Reports on the counteraction of barbiturate poisoning by succinate, pyruvate, and similar substrate molecules have been in many instances diametrically opposed to one another. Soskin and Taubenhaus (1943) reasoned from the work of Jowett and Quastel (1937) that succinate should maintain the brain metabolism in a barbiturate poisoned dog. Their results substantiated this contention. A year later, Beyer and Latven (1944) reported that while sodium malonate and glutamate were ineffective, sodium succinate did diminish the duration of pentobarbital anesthesia in mice and rats. The degree of reduction was not as great as had been reported by Soskin and Taubenhaus (1943). The fact that malonate did not prolong barbiturate anesthesia caused Beyer and Latven to suggest that succinic utilization alone was not normally a major factor in hypnosis. It would have been interesting to extend this work to see whether or not a hypnosis produced *in vivo* in the presence of comparatively large amounts of barbiturates and succinic acid would be prolonged by malonic acid. In other words would the effect of succinate in diminishing the duration of anesthesia be reversed or nullified by the simultaneous administration of malonic acid?

Further confirmation of the analeptic effect of succinic acid in barbituric acid poisoning came from Pinschmidt et al. (1945) and DeBoer (1946). The latter author found intravenous succinate superior to intraperitoneal

injection for counteracting pentobarbital anesthesia, and correlated the degree of diuresis produced by the chemical with its antagonistic potential for the barbiturate.

Corson, Koppanyi, and Vivino (1945) obtained no lifesaving effect through the use of sodium succinate or fumarate in dogs, cats, rabbits, or rats poisoned with short- or long-acting barbiturate. Furthermore, they observed no significant shortening of duration of anesthesia. Additional negative results were obtained by Schack and Goldbaum (1949).

The only clinical report to date is that of Barrett (1949) who found succinate an effective analeptic for barbiturate poisoning. In the broadest sense, the pyruvic acid antagonism of barbiturates can be said to support the succinic acid work. Westfall (1946) by the intravenous injection of pyruvic acid decreased the depth of depression, shortened the sleeping time but did not materially alter the total depression time in barbiturate treated rabbits.

The probability that the action of narcotics is associated with inhibition of the carbohydrate metabolism of the brain gains further support from observations on the relationship of thiamine to barbiturate anesthesia. The involvement of this vitamin in carbohydrate metabolism is well known. The first recorded work (Higgins and Mann, 1943) involved the use of a diet free of vitamin-B complex. The average period between injection of barbiturate and awakening was 28.8 minutes for rats on a standard diet, 67.7 minutes when the animals were on a vitamin-B complex deficient diet. The figure was 26.6 minutes when the test animals received the vitamin-B complex deficient diet properly supplemented with B factors. While this work involved the simultaneous elimination from the diet of all B-complex factors, it is probable that the natural cocarboxylase form of thiamine played a role. DeBoer (1948) found that simultaneous administration of thiamine actually increased the toxicity of barbiturates. For example, the LD-50 of pentobarbital sodium in rats was 90 mg./kg. while for thiamine injected animals the value was 80 mg./kg. Considering the capacity of barbiturates to inhibit phosphorylation mechanisms (Eiler and McEwen, 1949), it is possible that the thiamine in the experiments of DeBoer (1948) could not be phosphorylated and it seems entirely probable that thiamine could act as a biological antagonist of cocarboxylase.

Another line of evidence adding fuel to the antagonism concept of barbiturate action was initiated by Green and Musulin (1941). These investigators were able to correlate the vitamin-C level with the response produced by phenobarbital or pentobarbital in the guinea pig. The higher the vitamin level the less the depression produced by barbiturates. A correlative was the observation that guinea pigs on a diet low in ascorbic acid manifested a greater duration of susceptibility to barbiturate action (Richards et al., 1941). Whether or not barbiturates cause depletion of tissue

reserve of ascorbic acid is a matter of disagreement (Frommel et al., 1945; Green and Musulin, 1941) but this does not alter the picture from the standpoint of biological antagonism.

The second of the converging lines of research was the demonstration that pentobarbital inhibited the oxidative metabolism of brain *in vitro* at the flavoprotein-cytochrome b stage (Greig, 1946). Then, in 1947, Greig reasoned that the barbiturate block could be circumvented by introducing into the system some compound with the proper redox potential which could be reversibly oxidized and reduced. Ascorbic acid was the key and its effect in reducing the inhibition by pentobarbital of brain carbohydrate oxidation was potentiated by cytochrome c.

All of these threads woven together indicate blockage of carbohydrate metabolism by biological antagonism as the basis of action of barbiturates. The specific point of antagonism and the identity of the nutritive primarily displaced must await further work but the probability is that displacement of some B-complex factor from its function as a coenzyme may well be the answer.

That barbiturates have actions other than the key one of inhibition of oxidation mechanisms is to be anticipated. No drug has absolute specificity—everything in the pharmacological world is relative. In keeping with this concept, the barbiturates inhibit cholinesterase (Heim and Rohde, 1943; Schutz, 1943), and block the normal oxidation of alanine by the kidney (Persky and Levine, 1949). This latter observation gave rise to the proposal that barbiturates act by blocking pyruvate oxidation with consequent failure of acetate formation which would be essential in the formation of acetylcholine. While this hypothesis must be considered, it appears that the inhibition of cholinesterase by these drugs would tend to counteract their function in preventing acetylcholine biosynthesis. In any event, the theory could be fitted equally well into the pattern of biological antagonism. Furthermore, if the formation of acetate from pyruvate is the blocked step, then more than ever cocarboxylase is implicated.

Two additional phases of barbiturate pharmacology must be considered relative to the application of the concept of biological antagonism, namely the metabolism of the barbituric acid molecular form and the known pharmacological antagonisms.

With few exceptions, the barbiturates are metabolized in the liver and thus detoxified (Maynert and van Dyke, 1949). Several channels of metabolism are known, namely, oxidation of an alkenyl or alkyl group in the 5-position, dealkylation of N-alkyl molecules, and in the case of the 2-bromallyl group hydrolysis of the bromine. The importance of the liver in detoxication has been demonstrated by using animals poisoned with carbon tetrachloride and noting the prolonged duration of effect of the barbiturate (Shideman et al., 1947). The enzymes involved in the detoxication proc-

esses are not known but it is clear that the principles of biological antagonism could be applied to alter the speed of detoxication with resultant extension of the duration of effect of a given barbiturate. It is probable that in every instance, except those in which the drug is excreted unaltered, modifications of pharmacological activity could be achieved by blockade of the detoxication channels.

There are many known pharmacological antagonists of the barbiturates: picrotoxin (Maloney et al., 1931), coramine (Reese, 1933), caffeine (Reese, 1933), Metrazol (Zipf et al., 1937), and strychnine (Simon, 1937). They are mentioned here because it is probable that in the long run they will be fitted into the picture of biological antagonism.

Morphine and Related Molecules

The purpose in examining the morphine group of narcotics is to determine if the mechanism of action is similar to or identical with that of the barbiturates. At the outset, it is assumed that no difference exists.

Quastel and Wheatley (1932) noted the inhibition of oxygen consumption by cerebral tissues resulting from the addition of 0.12 per cent of morphine. This was a general phenomenon associated with narcotics. In fact, they defined narcosis as a depression of the normal functional activity of the nervous center. The findings of Quastel and Wheatley (1932) were confirmed and extended by Shideman and Seevers (1941) and Seevers and Shideman (1941) who reported the blockage by morphine of the activity of lactic, citric, and glucose dehydrogenases. Succinic and ethanol dehydrogenases were not affected. Codeine and thebaine were inactive. It is interesting to note that these investigators implicated cocarboxylase in the mechanism of action of morphine. In their experiments, the increase in the oxygen uptake of normal muscle resulting from the addition of cocarboxylase after pyruvate was entirely lacking in both thiamine deficient and chronically morphinized muscle.

While in general succinic dehydrogenase has been considered to be narcotic insensitive, both Sen (1931) and Watts (1949) have reported its inhibition by these drugs. The latter author found that it required two to five times as much of the narcotic to block the succinic system as it did for the inhibition of the oxidation of glucose, lactate, or pyruvate. With this quantitative factor for consideration, it seems probable that larger amounts of barbiturates would also block succinic dehydrogenase. Here, we see another manifestation of biological relativity; succinic dehydrogenase is only relatively insensitive to the blocking effect of narcotics. Watts from his own work and that of Ball et al. (1947) and Slater (1948) concludes that methadon blocks at the cytochrome c-cytochrome oxidase level.

A striking contribution to the concept of differential tissue requirements for enzymatic activity is that of Shideman and Seevers (1941, 1942) who

observed that the in vitro addition of morphine to muscle tissue of rats increased the oxygen uptake. This is in marked contrast to the effect of the drug on brain tissues. In chronically morphinized rats, the elevated oxidative metabolism of the muscle tissues is maintained when the morphine is

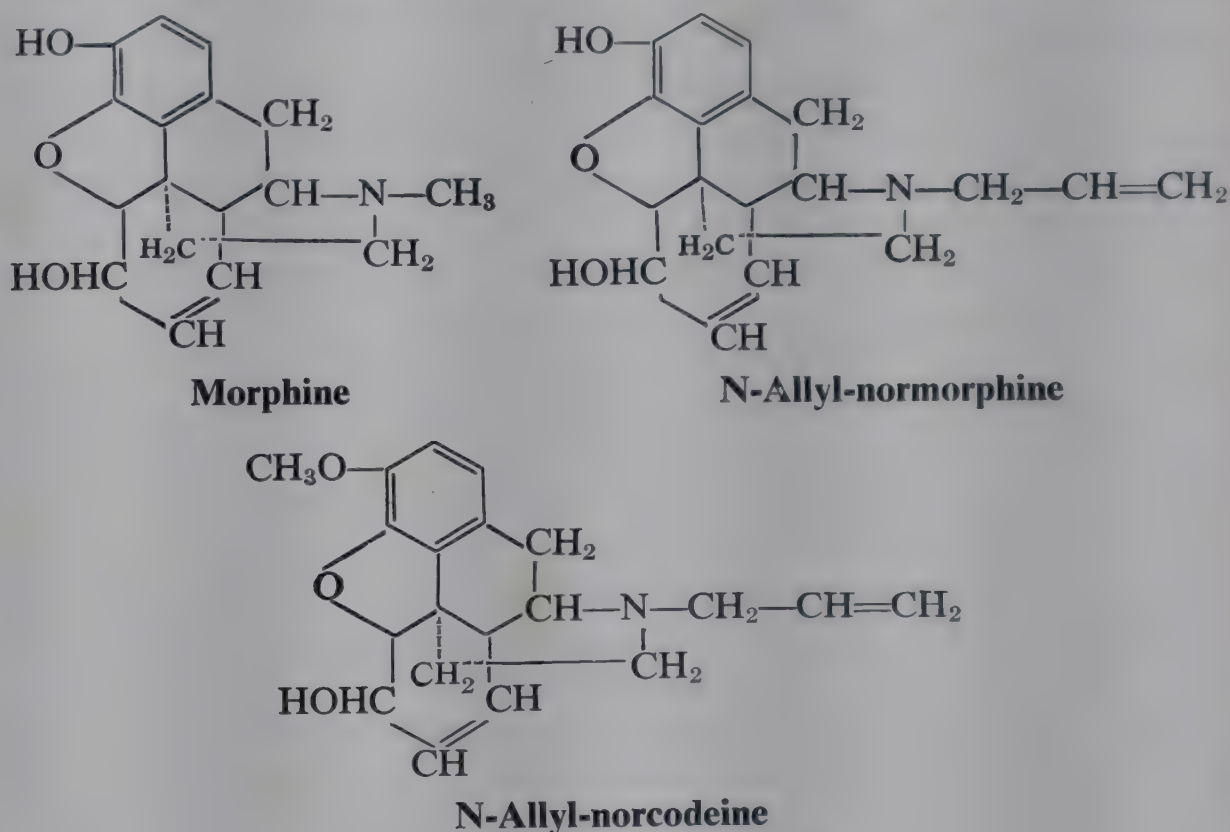


FIG. 9. Morphine and derivatives.

withdrawn. The phenomenon of stimulation of enzymatic activity is usually associated with the blockage of the action of an enzyme which antagonizes the first. This antagonism may be indirect rather than direct in the sense that one enzyme destroys the substrate utilized by a second: hence, if the first enzyme is inhibited, the second will be stimulated or accelerated in its activity.

It is probable that observations such as those of Keeser (1932, 1933) are incidental to the main theme of narcotic action but they do disclose other activities of these agents. Pepsin, trypsin, erepsin, cathepsin, phenolase, and pig-liver esterase were not affected by morphine; however, at certain concentrations, invertase, tyrosinase, phosphatase, lecithinase, and urease were inhibited.

Biological antagonism has been demonstrated within the series of morphine relatives. In 1915, Pohl noted that N-allyl-norcodeine antagonized the respiratory depressant action of morphine. In one experiment, a dog was aroused by the drug from a deep sleep induced by morphine. Later, McCawley et al. (1941) and Hart (1943) found N-allyl-normorphine to be more powerful than N-allyl-norcodeine in blocking the respiratory de-

pression caused by morphine. Unna (1943) confirmed and extended the observations of these workers. He reported that N-allyl-normorphine prevented or abolished the action of morphine; it blocked the analgesia induced by morphine in mice, the morphine respiratory depression in rabbits, the general depressant action of the alkaloid on dogs and its stimulatory effect on cats.

Reports have been made on other narcotics and related substances which can be correlated with the general oxidative metabolism depression of the brain by barbiturates and morphine. Chloretone, cocaine, and ethylurethane have an identical action (Michaelis and Quastel, 1941; Sherif, 1930; Johansson, 1931). Mescaline (Schueler, 1948) not only parallels the effects seen with the previously mentioned narcotics in that it depresses respiration on lactate, pyruvate, and glucose but in addition, succinate is an effective antidote for mescaline in human subjects.

A complicating factor in allocating narcotic action to those chemicals with the capacity to reduce brain oxidative mechanisms lies in the probability that picrotoxin (Klein, 1943) which is a powerful stimulant to the central nervous system also depressed these same oxidative channels. Furthermore, these findings do not fit into the picture presented by Krantz et al. (1937) in which they found that picrotoxin antagonized the depression of oxygen consumption produced by barbiturates in rats. It is probable that the action of picrotoxin on isolated brain is one of phase and that Klein (1943) operated in the inhibitory phase of picrotoxin concentrations.

Just as the barbiturates inhibit cholinesterase, so morphine is an active inhibitor (Bernheim, 1940; Slaughter and Gross, 1940). There is, however, no correlation between relative activity as cholinesterase inhibitors and effect on different tissue in the series of morphine derivatives (Gruber and Brundage, 1935). An exception has been made in that there does seem to be a correlation between anticholinesterase activity and central emetic power of morphine compounds (Kuhn and Surles, 1938).

Concerning the metabolism of morphine and possible applications of biological antagonism, it is known (Oberst, 1940) that a large percentage of morphine is excreted in the urine in a conjugated form and the probability is that it is as a glucuronide (Bernheim and Bernheim, 1945). Assuming confirmation of this report, it is apparent that any drug inhibiting glucuronide formation would modify the pharmacological activity of morphine. Additional support for this contention comes from the observation that morphine tolerant dogs and human beings destroy more of the drug and excrete less in the urine as a conjugate (Oberst, 1940).

Miscellaneous

Clark (1937) reviewed those instances in which drugs were found inhibitory to enzymatic activity: cyanides inhibit oxidases; atoxyl and quinine

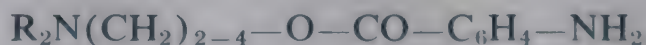
inhibit lipases; cocaine, atropine, and pilocarpine inhibit yeast invertase. These and many other instances are cited but we refer here only to illustrative examples.

The probability that structurally similar breakdown products of drugs might displace a drug from an action site has been established by Richards (1945) and Richards and Kueter (1946) who found that the split products of procaine altered the typical convulsive action of that drug in guinea pigs. Both *p*-aminobenzoic acid and diethylaminoethanol were effective in reducing the incidence and severity of the convulsions. The quantitative relationships between procaine dosages and those of the split products strongly suggest competitive inhibition. A mixture of the two antagonists, *p*-aminobenzoic acid and diethylaminoethanol, was most effective as tested. The observed effect was probably not due to the inhibition of procainesterase as Kisch (1943) has shown that the convulsive action of procaine was not modified by the administration of eserine which is a known inhibitor of the enzyme.

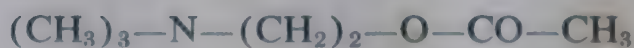
The competitive inhibition was relatively specific since *o*-aminobenzoic acid was inactive. Again, *p*-hydroxybenzoic acid showed moderate activity and salicylic acid none. Benzoic acid was the antagonist second in power to *p*-aminobenzoic acid. It was stated by Richards and Kueter (1946) that any deviation from the *p*-aminobenzoic acid molecule decreased the efficiency of the molecule as a protector.

With the aminoalcohols, there was the same close relation between antagonistic power and chemical constitution. Diethylaminoethanol was the most powerful but an increase in chain length by one methylene unit did not materially alter the protective efficacy. It is remarkable that the protective action of both split products was directed against convulsions produced by procaine and not against the local anesthetic action of the drug. In this same vein, the sulfonamide molecule has been reported to possess the power of reducing the local anesthetic effect of procaine (Hazard, 1944; Hazard et al., 1947, 1948).

Thimann (1943) has offered a hypothetical concept with experimental substantiation that the structures of the known local anesthetics resemble that of acetylcholine. The action of the anesthetic is supposedly due to its ability to compete with acetylcholine for the receptive substance at nerve endings. This receptive substance is the reactive site of the parasympathetic effector cells which is the normal contact point. The structures of the local anesthetics and of acetylcholine do show marked similarities.



Local anesthetic



Acetylcholine

Substantiation of the concept of local anesthetic action as a manifestation of biological antagonism came from the observations of Rapp (1947) who reported that procaine blocked the reaction between acetylcholine and phosphocreatine, hence preventing the release of energy required for the transmission of nervous impulses.

In general, we have been considering those pharmacological agents the action of which is modified either by immediate displacement or by alteration in the kinetics of enzyme functioning in the formation or destruction of the drug. Beyond this there is a great class of drugs which become active by virtue of enzymatic function. Of these, it is our purpose to consider only one, acetanilide. This antipyretic normally functions as the substrate of an enzyme system, the product of which is p-aminophenol. The reaction is catalyzed by an acylase (Michel et al., 1937). While p-aminophenol shows a marked capacity to inhibit oxygen uptake by tissue suspensions (Baumgärtel, 1943), its oxidation product, a quinimine, has no similar effect on oxidative mechanisms of a number of compounds added to liver suspensions. The exceptions are xanthine and phospholipids (Bernheim, 1939; Bernheim and Bernheim, 1938). Quinimine inhibits completely the xanthine oxidase of liver and guanase. In this pharmacological system, the antipyretic, acetanilide, is hydrolyzed by an acylase to aniline, oxidized to p-aminophenol and in turn to a quinimine. These metabolic products of enzymatic action function by virtue of enzymatic blockage doubtless through displacement.

Study of this sequence focuses attention on the necessity for the consideration of all phases in the metabolism of a given molecule. Pharmacological activity may be related to some distant metabolite. Biological antagonisms could enter this picture by preventing the formation of the active metabolite, by preventing its destruction or by displacing the pharmacologically active metabolite itself.

That we may indicate the scope of the application of the enzymatic inhibition concept to pharmacological activity, it seems in order to mention a few miscellaneous instances of record. Mann and Keilin (1940) found that sulfanilamide specifically inhibits carbonic anhydrase. In the same study, they observed no inhibition of cytochrome oxidase, uricase, phenolase, or catalase. The blocking effect was attributed to chelation of zinc by the drug. As another example, it has been proposed that the activity of salicylates is associated with their ability to stimulate the activity of citric acid oxidase (Michel et al., 1937).

Biological antagonism doubtless constitutes the basis of such pharmacological antagonisms as those manifested between ethylenimonium and sulfonium cations under certain conditions (Gilman and Philips, 1946). In fact, the very nucleotoxic action of nitrogen and sulfur mustards will probably be based upon irreversible enzyme inactivations or nucleic acid modification with resultant blockages.

Other isolated instances of pharmacological antagonisms doubtless associated with displacement phenomena include: sodium diphenylhydantoinate and nikethamide (Granier-Doyeux, 1946), prostigmine homologues and tubocurarine (Bülbring and Chou, 1947), nicotine and sodium sulfathiazole or thiamine HCl (Pick and Unna, 1946), sulfonamides in general and nicotine (Pick et al., 1944), D-tubocurarine and decamethylene-bis-(trimethylammonium bromide) (MacFarlane et al., 1950) and epinephrine and acetylcholine.

The natural antagonists of pharmacological agents must also be considered. Thiamine will inhibit the action of nicotine on the isolated intestine of the rat (Unna and Pick, 1944). This same effect is manifested by cocarboxylase. As another example, Lecoq et al. (1946) studied the positive effect of purines such as caffeine and theobromine on neuromuscular and cardiac excitability as reflected in chronaximetry and found that the oral or parenteral administration of sugars—glucose, galactose, sucrose, lactose—materially reduced the effect. Further detail on the influence of various vitamins on the nervous effects of pharmacological agents including epinephrine, acetylcholine, choline, and adrenochrome has been presented by Chauchard et al. (1948).

Comment

Pharmacology is applied biological antagonism. It has an enzymatic basis; metabolite analogues play a major role. In some instances, true competitive displacement occurs; in others, the mechanism is noncompetitive or even irreversible. The toxic effects of drugs represent quantitative but not qualitative differences. Above all else, the entire structure is relative; it is another manifestation of the general principle of biological relativity.

Tissue selectivity or pharmacological specificity is a reflection of the summation of enzyme concentrations and of reaction sites on effector cells. There seems no reason to regard the reaction sites on effector cells as being different in any fundamental way from those on enzyme surfaces. The kinetics of enzymatic activity and of enzyme inhibition apply equally to pharmacological activity phenomena.

The complexity of pharmacological reaction is great and consideration must be given not only to concentration of drug but also to concentration of metabolic products. The effective concentration of a drug will depend upon its absolute concentration at a given time and upon the amounts of natural and synthetic inhibitors present. Biological variation in response to drugs will be mirrored to a degree in the inhibitor phase. Furthermore, the absolute concentration of a given drug will be a function of the speed of formation or supply and the speed of metabolic destruction. In some instances speed of enzymatic destruction of a drug would tend to prevent toxicity, i.e., monoamine oxidase kinetics and epinephrine toxicity; in others

the speed of metabolism might result in excessive formation of a more toxic material, that is, acetanilide might be too rapidly metabolized to p-amino-phenol with resultant toxicity. Williams (1947) has stated that there are two ways in which a so-called detoxication mechanism can produce toxic agents in the body: by conversion of the foreign compound, usually by oxidation or reduction into toxic agents; and by conjugation of the chemical with an essential metabolite, with resultant deprivation of the body of the metabolite for normal nutrition. He concludes that in general the metabolic fate of a foreign molecule will parallel that of the natural metabolite to which it is most closely related. For example, if the foreign compound happened to be an α -amino acid, it would fit as a key into the processes of oxidative deamination or acetylation just as the natural amino acids form substrates for these reactions. Biological antagonism enters this system in that such enzymatic reactions will preferentially attack either foreign molecule or natural substrate to the exclusion of the molecule of less affinity for reaction site.

Inhibition of the conjugation of epinephrine with sulfate which results in its inactivation (Richter, 1940) would clearly increase the duration of action of this drug, assuming there were no compensatory elevations in the activities of either monoamine oxidases or phenolases. Blocking the acetylation of sulfonamides and turning the "detoxication" reactions into the channel of glucuronide formation would render sulfonamides safer to use (Scudi, 1940).

Drug tolerance and bacterial resistance have been regarded as separate entities. This position must be abandoned as both are manifestations of enzymatic adaptation. While this has long been regarded as a common phenomenon in bacterial systems (Yudkin, 1938), it has only recently been accepted as applicable to physiology and pharmacology. Its acceptance was based upon work such as that of Lightbody and Kleinman (1939) who demonstrated by repeated administration an increased power of the kidney to oxidize alcohol; and that of Fishman (1940) who observed increased glucuronidase activity in various tissues resulting from continued dosage with menthol or borneol. While the general subject of drug tolerance and bacterial adaptation will be reviewed in some detail in Chapter 21, it is mentioned here in the interest of correlation of biological antagonism and pharmacology.

Similarity of detailed structure among enzyme reaction sites and effector cells doubtless accounts for pharmacological overlap; e.g., atropine is an effective displacer of acetylcholine both from a parasympathetic system effector cell site and from cholinesterase. The affinity is greater for the effector cell and hence the drug acts as an antispasmodic.

The complexity of the action of pharmacological agents is often mirrored in the influence one has on the toxicity of another. It is probable that in all

cases this is due to the action of one agent as an inhibitor of the activity of an enzyme acting on the second. For example, physostigmine increases the toxicity of procaine by functioning as an inhibitor of procainesterase which would normally destroy the drug by hydrolysis.

Another factor in the study of biological antagonism as applied to pharmacology resides in the structural differences of effector cell reaction sites within the same system. This is exemplified by the specificity of anti-acetylcholine drugs for effector cell sites at neuromuscular junctions, ganglia, and nerve fiber. Nature has apparently directed the creation of a system of balance and counterbalance within each physiological system and one such balancing knife edge is the dissimilarity of effector cell sites which would contribute to parasympathetic nervous system stability by rendering natural and synthetic inhibitors of one effector cell inactive against another.

An extension of the overlap phenomenon is seen in the existence of drugs acting on the inhibitor or excitor sympathetic components or, as in the case of epinephrine, on both. Here, as with the parasympathetic system, effector cell reaction sites must vary in their detailed structure.

The fact that antihistamines possess in varying degree the characteristics of local anesthetics, antispasmodics, sympathomimetics, and sympatholytics tends to underline the probable structural similarity of the receptor sites for histamine, acetylcholine, and epinephrine.

Destructive enzymatic activity for a given pharmacological agent is generally multiple in nature. There is not only one catabolic channel for epinephrine, there are actually three known mechanisms via monoamine oxidase, phenolase, and sulfoesterase. Again, we see biological relativity manifested; it is biological relativity that gives balance and stability to the living organism by providing few if any absolute channels of metabolism.

In recent years and largely through the effort of Szent-Györgyi (1949) the contractile structure of muscle has been demonstrated to consist of a protein complex, actomyosin, which is composed of myosin and actin. Neither protein in itself nor actomyosin shows any sign of contractility without the key substance, the water-soluble nucleotide, adenosinetriphosphate. The adenosinetriphosphate is not merely the source of energy for muscular contraction but actually dominates the entire physical state of muscle.

Direct observations of the action of pharmacological agents on actomyosin-ATP complex are now possible. Such studies point the pathway to the future. One approach has been made by experiments on the inhibition of the adenosinetriphosphatase activity of preparations of myosin (Binkley, 1949). He noted the inhibition of the ATPase by posterior pituitary and the reinforcement thereof by acetylcholine. Acetylcholine alone possessed no inhibitory activity. Theoretically, inhibition of ATPase should result in a contracted state of the myosin complex and if this is found to be the case

the results would be consistent with the known pharmacological effects of acetylcholine and the principles of the posterior pituitary.

The most recent work of this type is that of Snellman and Gelotte (1950) who in the course of isolating heart actin found a contaminant, a deaminase, which acted on adenosinetriphosphate and prevented the polymerization of the actin. Turning to cardiac glucosides, these workers found that the use of such drugs prevented the action of the deaminase. In the absence of the glucosides, they were able to dialyze out of the actin an inosine derivative but if the actin had been treated with the drug they obtained an adenyl derivative on dialysis.

Snellman and Gelotte (1950) also confirmed the results of Horvath, Kiraly, and Szerb (1949). Both groups demonstrated that a cardiac glucoside has a direct effect on the polymerization of actin. The drug facilitates the polymerization of the protein to a great degree and the authors conclude that a major portion of the effect of such agents lies in their prevention of the destruction of actin.

Other studies utilizing the actomyosin complex or portions thereof as test systems for pharmacological agents have been made. One of these is the report of Straub et al. (1948) who found that the polymerization of globular actin varies with different ratios of calcium chloride and potassium chloride at 24° C. and pH 7. Epinephrine (0.1 mg./ml.) greatly enhances the reaction. Quinine, strychnine, and veratrine inhibit at the same concentration.

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Chapter 3

p-AMINOBENZOIC ACID, SULFONAMIDES, FOLIC ACID

1. p-Aminobenzoic Acid and the Sulfonamides.
 - a. Specificity of p-Aminobenzoic Acid in Counteracting Anti-bacterial Agents.
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2. Secondary Inhibitors of Sulfonamides.
 - a. Methionine.
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 - c. Nicotinic Acid and Amide.
3. Mechanisms of Action of Sulfonamides Explained on Bases Other than that of p-Aminobenzoic Acid Displacement.
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4. Folic Acid Synthesis as the Site of Sulfonamide Action.
 - a. Basis of the Concept.
 - b. Limitations of the Concept.
 - c. Explanation of the Limitations Based Upon the Existence of Various Forms of "Folic Acid."
 - d. Involvement of Purine and Amino Acid Metabolism.
5. Recapitulation.

This relationship, its study and extensions, formed the basis for the revival of interest in biological antagonism occurring over the past ten years. McIntosh and Whitby (1939) thought that sulfanilamide acted by blocking the food supply of bacteria through enzymatic inactivation. During the same year, Stamp (1939) extracted a substance from *Streptococcus* which antagonized the action of the sulfonamides. He suggested that this material was a nutrient, the formation of which was prevented by the drug. Green (1940) extended the work of Stamp, also isolating a sulfonamide inhibitor from bacteria. It remained for Woods (1940), who had obtained the antagonist from yeast, to identify the compound as p-aminobenzoic acid, PABA.

It is not intended to review the reports which have accumulated concerning the PABA-sulfonamide relationship, as this has been done by Henry (1944) and by Northey (1948); presented here, in the interests of continuity only, are the salient features of this vast literature.

p-Aminobenzoic Acid and the Sulfonamides

In accordance with the concept of the relativity of biological antagonism, PABA is not solely an inhibitor of the action of the sulfonamides (Miller, 1941), possessing as it does a capacity to reverse the antibacterial effect of sodium p-nitrobenzoic acid. Another example of the lack of specificity of PABA is its efficacy in preventing the chemotherapeutic action of certain arsenicals (Peters, 1943; Hirsch, 1942) such as sodium p-aminophenyl-arsionate. It is not effective against any of the arsenoxides, e.g., mapharsen.

Furthermore, PABA with its structural similarity to the p-isomer of the sulfanilamide group is also inhibitory to the other isomers (Sevag et al., 1943). Even the selenium and tellurium analogues of sulfanilamide are susceptible to reversal by the aromatic amine (Green and Bielschowsky, 1942). Moller and Schwartz (1941) extended the list of chemotherapeutic agents counteracted by PABA when they added germanin and Neostibosan, neither of which bears any structural similarity to the aminobenzoic acid. 4,4'-Diaminobenzil, reported to be twice as active as sulfanilamide against *Staphylococcus pyogenes*, was antagonized by PABA (Kuhn et al., 1943). Another molecule, 4,4'-diaminobenzoin, studied in this same series, responded identically.

Other chemotherapeutic agents counteracted by PABA were reported by Auhagen (1942). He included p-aminacetophenone, p,p'-diaminobenzophenone, and p-aminobenzophenone in the group. The fundamental mechanisms which make one compound an inhibitor of the antibacterial action of another could not bear 100 per cent specificity in relationship. In each instance, the inhibiting molecule would be an effective agent against other chemotherapeutic molecules. This is of vital significance in the rational application of the entire concept of biological antagonism. It is another manifestation of biological relativity.

While the action of PABA as an antagonist extends to divalent sulfur compounds of the type of 4,4'-diaminodibenzenedisulfide, it does not act against the simple oxidation product of sulfanilamide, p-hydroxylaminobenzenesulfonamide (Green and Bielschowsky, 1942). There are many examples of the PABA inhibition of antibacterial agents which are close structural analogues (Johnson et al., 1944; Martin and Rose, 1944). 2-Chloro-4-aminobenzoic acid (Johnson et al., 1944) is the best example of such molecules; it shows bacteriostatic action at high concentrations and typical PABA effects at lower concentrations.

Just as PABA lacks specificity in inhibiting the chemotherapeutic activity of agents other than the sulfonamides, so it fails to inhibit all of the actions of the sulfonamides. An outstanding example of this is its failure to counteract the toxic effects of sulfathiazole therapy in the human being (Strauss et al., 1941) as manifested by fever and rash. In these same patients, the

aromatic amine was capable of completely counteracting the bacteriostatic action of the drug in the blood.

The great complexity of the interrelationship of PABA and the sulfonamides is extended by studies on the counteraction of sulfonamide inhibitors. Azochloramid (Schmelkes and Wyss, 1942), urea (Tsuchiya et al., 1942; Wallersteiner, 1943), and allantoin (Wallersteiner, 1943) have been reported to counteract the action of sulfonamide inhibitors. These findings do not hold for all bacteria (Stokinger et al., 1942; Kirby, 1943). In fact, the results with urea are questioned *in toto* (King and Strangeways, 1942). The report of Wallersteiner (1943) casts an entirely new light on this general problem. He found that γ -methyl- α -indole- β -aminopropionic acid added to cultures of sulfonamide-fast streptococci greatly increased the production of inhibitor, whereas the production of this antagonistic principle

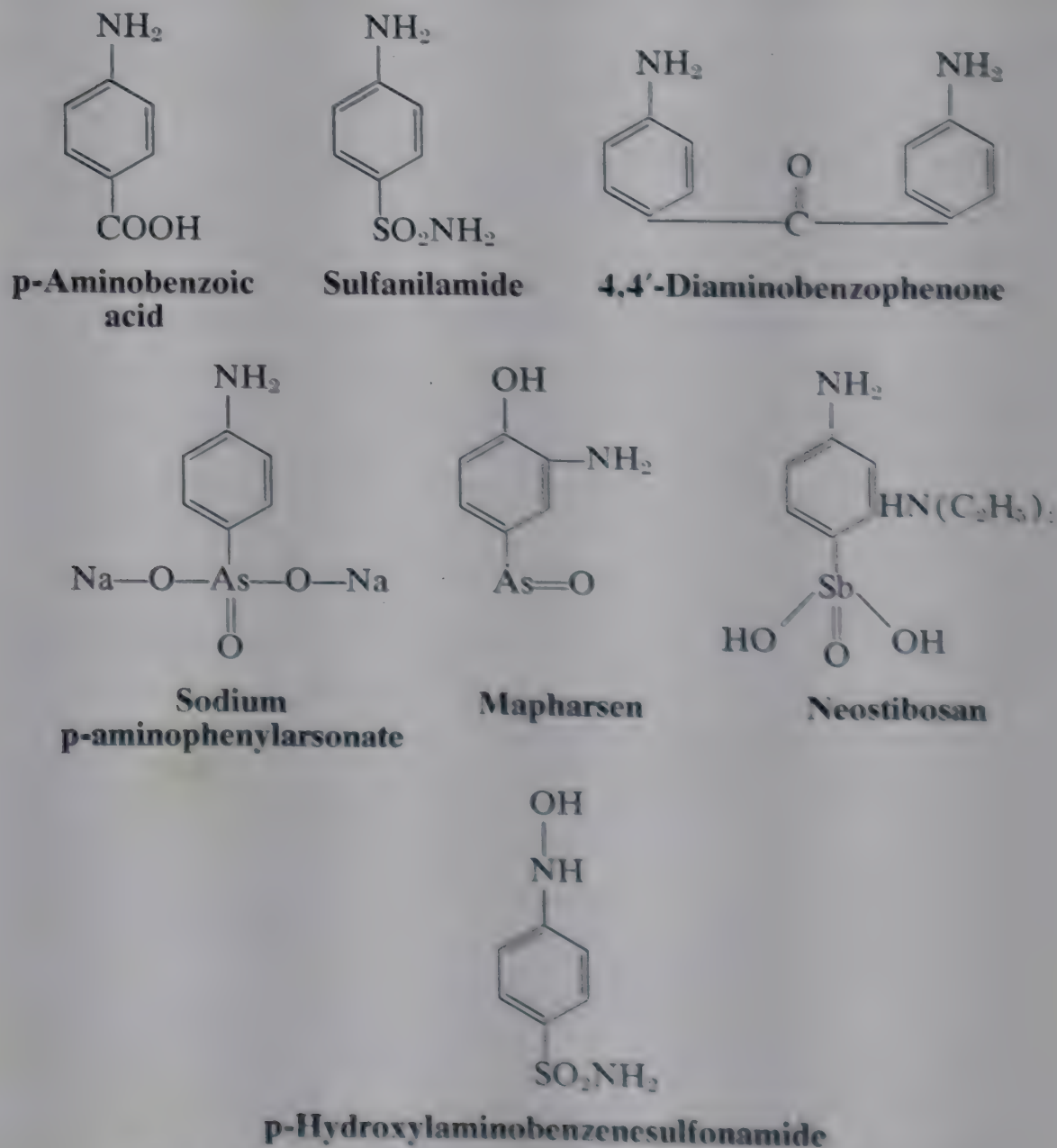


FIG. 1. Antibacterial agents studied as PABA antagonists.

was greatly retarded in media poor in tryptophan and organic sulfhydryl. The extreme difficulty of accurately analyzing results of this sort can be understood when it is considered that investigators differ markedly in their interpretation of the mechanisms underlying sulfonamide resistance. There has been no work actually showing decreased production of a specific chemical inhibitor of the sulfonamide drugs under the influence of another chemical such as urea. The reported observations could represent the summation of effects of two agents capable of affecting the metabolic processes of bacteria under the conditions of the experiments.

A logical explanation of the above phenomena backed by laboratory evidence has been presented by McClintock and Goodale (1943) who found that urea blocks the combination of sulfanilamide and albumin. This combination if formed renders the sulfanilamide inactive. The apparent synergism between urea and the sulfonamides is therefore not concerned with p-aminobenzoic acid, although it would be interesting to determine the effect of PABA itself on the formation of protein-sulfonamide combinations. Theoretically, PABA should block the formation of such complex units. The potentiality remains that the urea action is nothing more than a manifestation of its own bacteriostatic power. In this connection, Kirby (1943) reports that concentrations of urea which in themselves are not bacteriostatic exert no potentiation of sulfonamides nor do they counteract sulfonamide antagonists.

A logical extension of the concept of structural displacement and the PABA-sulfonamide interrelationship was the proposal that susceptibility of bacteria to sulfonamides varies with their ability to synthesize PABA (Green, 1940; Green and Bielschowsky, 1942; Landy et al., 1943). The work of Landy and his group (1943) stresses their belief that while PABA synthesis explains the sulfonamide resistance of *Staphylococcus aureus*, it does not explain that of *Escherichia coli*, *Vibrio comma*, *Shigella dysenteriae*, or *Diplococcus pneumoniae*. A complicating factor in this consideration lies in the fact that there is a marked difference in the behavior of sulfonamide inhibitors in the test tube and in the tissues of living animals (Reed et al., 1944). This conclusion is based upon the demonstration that inhibitor production by *Clostridium* in vitro explains susceptibility to sulfonamides, and on a subsequent observation that sulfonamide susceptibility in vitro and in vivo were not parallel phenomena.

Secondary Inhibitors of Sulfonamides

Kohn and Harris (1943) first elaborated the theory of secondary inhibitors. These agents are noncompetitive antagonists and can be classified according to Work and Work (1948) into four major groups: essential metabolites which are displaced; substances which increase growth rate and so mask sulfonamide action; compounds which combine with sulfonamides;

and metabolites secondary to some prime reaction inhibited by the drug. Actually, the last named are the true secondary inhibitors. A large number of secondary inhibitors are known and the list is presented in Table I.

Table I
SULFONAMIDE INHIBITORS

Compound or Substance	Test System	Comment	Reference
Adenine	<i>Lactobacillus arabinosus</i> , <i>L. pentosus</i>	Presence of suboptimal amounts of PABA	Snell & Mitchell, 1942
Guanine	"	"	"
Xanthine	"	"	"
Hypoxanthine	"	"	"
Glucose	Bacteria		Broh-Kahn, 1939
Xylose	<i>Serratia marcescens</i>		Illenyi, 1943
Mercuric chloride . . .	Bacteria		Lamanna & Shapiro, 1943
Urethane	Luminescent bacteria		Johnson, 1942
Coccarboxylase	<i>Staphylococcus aureus</i> , Yeast		Sevag et al., 1945
Sodium pyruvate	<i>Staphylococcus aureus</i> , Yeast		Sevag et al., 1945a
Nicotinic acid	<i>Lactobacillus arabinosus</i>	Sulfapyridine only	Teply et al., 1943
Nicotinamide	<i>Staphylococcus aureus</i> , <i>Shigella</i>	Nicotinamide stimulated respiration	Dorfman & Koser, 1942; Wood & Austrian, 1942
Coenzyme I	<i>Staphylococcus aureus</i>	Sulfapyridine only	West & Coburn, 1940
Pantothenic acid	Rat	Sulfapyridine only	West et al., 1943
2-Chloro-4-amino-benzoic acid	<i>Streptococcus hemolyticus</i> , <i>Escherichia coli</i>		Johnson et al., 1944
p-Aminobenzoyl-l-glutamic acid	"		Johnson et al., 1944; Auhagen, 1943; Williams, 1944
p-Aminocinnamic acid	"		Johnson et al., 1944
p-Aminobenzoic acid .	Bacteria		Woods, 1940
Peptides of PABA . . .	"		Blanchard, 1941
m-Aminobenzoic acid	"		Rubbo & Gillespie, 1940, 1941, 1942
o-Aminobenzoic acid .	"		" "
Methionine	"		Bliss & Long, 1941; Kohn & Harris, 1943
Arginine	"		Bliss and Long, 1941

Table 1—(Continued)

Compound or Substance	Test System	Comment	Reference
Lysine	Bacteria		Bliss and Long, 1941
Glycine	<i>Escherichia coli</i>	Presence of methionine	Kohn & Harris, 1943
Cozymase	"	"	Kohn & Harris, 1943; Spink et al., 1942; Wood & Austrian, 1942
Serine	"	"	" "
Allothreonine	"	"	" "
Guanine	"	"	" "
Xanthine	"	"	" "
Glutamic acid			Hill & Mann, 1942; MacLeod, 1940
Glutamine			" "
Bacteria			Green & Bielchowsky, 1942a
Plasma			McIlwain, 1942
Urine			MacLeod, 1940
Peptones			Lockwood & Lynch, 1940
Proteins			Henry, 1944
Necrotic tissue			"
Tissue extracts			"
Bacterial products			"

Results reported on studies with secondary inhibitors of sulfonamides involve the danger of inaccurate interpretation based upon a failure to recognize added growth factors, contamination by PABA, pH factors not adequately controlled, concentration factors imbalanced, inoculum size variations, alterations in temperatures, history of the bacteria used, composition of medium as a variant, or inconsistency in method for determination of growth rates. Any single deviation may completely nullify the value of the results obtained.

Methionine in the presence of inhibitory concentrations of sulfonamides can reinstitute secondary reactions by supplying them with missing substrates, the secondary antagonists (Kohn and Harris, 1943). These secondary inhibitors will vary as the metabolic systems affected are changed. There is little reason to expect that a secondary inhibitor of a sulfonamide in one bacterial system will perform the same function for another. If the test bacterium is staphylococci, secondary antagonists will be found which may not function at all when the testing is done using streptococci. There

is no question but that antagonism will be manifested by the precursors of the primarily inhibited enzymatic systems as well as by substrates and the products of the reaction. It is interesting to note that as a secondary inhibitor, methionine is ten times as effective in the L-form as it is in the D- (Harris and Kohn, 1941). The relative efficacy was determined in a system in which methionine itself gave no growth stimulation.

Strauss et al. (1941a) using *Escherichia coli* found that methionine would antagonize sulfanilamide but not sulfathiazole or sulfadiazine, and they concluded from this that the more complex sulfonamides inhibit loci in the cell other than those affected by sulfanilamide. Methionine prevents the development of sulfonamide resistance (Strauss et al., 1941a) and permits indefinite growth without acquisition of resistance (Harris and Kohn, 1941). It would seem that supplying the substrate essential for the secondarily inhibited system makes unnecessary the acquisition by the bacterium of methods circumventing the metabolic blockage.

The inhibition of carboxylase activity in the yeast cell by sulfathiazole has been reported by Sevag et al. (1945), who found that one molecule of cocarboxylase added to the reaction system counteracted the inhibitory effect of between 8088 to 53,400 molecules of sulfathiazole. This same action of cocarboxylase was demonstrated using *Staphylococcus aureus* as test organism. The explanation offered is that cocarboxylase and the sulfonamide compete for the specific carboxylase protein. In the second paper of the series, Sevag et al. (1945a) record observations indicating that sulfathiazole and sodium pyruvate compete for the active site of carboxylase. Presence or absence of inhibition depends on which of the two substances gets to the active site of the enzyme. p-Aminobenzoic acid failed to reverse the inhibition exercised by sulfathiazole on the carboxylase activities of *Escherichia coli* or *Staphylococcus aureus*. The third paper of the group (1945b) deals with the action of neopeptone in counteracting the strong inhibitory effects exercised on the carboxylase activity of *Escherichia coli* by the ortho-, meta-, and para- isomers of aminobenzenesulfonamides. The drugs combined reversibly with the enzyme proteins. The investigators suggest that foreign proteins may exercise their antisulfonamide action by combining with either enzyme proteins or drugs.

The failure of PABA to counteract the inhibition by sulfathiazole of the carboxylase system must indicate that carboxylase systems do not play an important part in growth; this in view of the fact that PABA inhibits the bacteriostatic action of sulfonamides. A somewhat similar problem was presented by Teply et al. (1943) when they found that sulfapyridine bacteriostasis of *Lactobacillus arabinosus* was completely counteracted by p-aminobenzoic acid or by higher levels of nicotinic acid. This multiplicity of attack-points by the more complex sulfonamides quite clearly indicates the probable value of the use of multiple inhibitor capacities in one mole-

cule, or of the administration of molecules with multiple inhibitor capacities in simple admixture. The introduction of the concept of multiple inhibitory activities of complex sulfonamides was originally made by Dorfman and Koser (1942) as a result of their observations that sulfapyridine and sulfathiazole, in contrast to sulfanilamide, inhibit the nicotinamide stimulated respiration of the dysentery bacillus when grown on a medium deficient in nicotinamide. This inhibition was not reversed by PABA. The growth inhibition was counteracted by PABA, but not the blockage of the nicotinamide stimulated respiration effect. Additional evidence of the overlap phenomenon as seen in the realm of the sulfonamides came with the work of West and Coburn (1940) who found that nicotinic acid did not have the reversing power of coenzyme I on the bacteriostasis produced by a complex sulfonamide.

Wood and Austrian (1942) emphasized the necessity of basing assertions concerning antisulfonamide activity upon the demonstration of such action in systems in which the antisulfonamide itself produces no growth effect. They had observed the direct proportionality between growth stimulant action and the ability of nicotinamide and cozymase to reverse the bacteriostatic effect of the sulfonamides against *Staphylococcus aureus*. The position of those investigators attempting to correlate the inhibition of nicotinic acid stimulated respiration with growth effects of sulfonamides is rendered untenable by the fact that acetylsulfapyridine, which has no effect on growth, inhibits this same respiratory mechanism (Koser et al., 1943; Wyss et al., 1942).

It seems clear that the more complex sulfonamides block enzymatic reactions other than those primarily associated with the metabolism of PABA. Such overlap may lead to an improved chemotherapeutic index or it may lead to toxicity as in the case of sulfapyridine. The suggestion may be advanced that the simpler structures in any given series of molecules will exert greater specificity but that in general greater specificity may be expected to correlate with diminished chemotherapeutic efficacy.

Mechanisms of Action of Sulfonamides Explained on Bases Other than that of p-Aminobenzoic Acid Displacement

Several lines of evidence exist indicating the probability that PABA displacement alone would not account for the action of the sulfonamides. One of these is the observation of Wallersteiner (1943) that γ -methyl- α -indole- β -aminopropionic acid greatly increased the production of inhibitor by sulfonamide-fast streptococci. Conversely, the antagonist production by the bacteria was retarded by the use of a medium poor in tryptophan. In the same year, Mirick (1943) observed and reported the bacteriostatic action of sulfonamides for soil bacteria requiring no PABA for growth. In fact, these bacteria actually destroy PABA. This would be a difficult finding to

integrate into the concept of competitive antagonism by the sulfonamide for an enzyme site.

Another line of evidence against the PABA displacement concept comes from the work of Snell (1943) who found that anthranilic acid was capable of replacing tryptophan in the metabolism of *Lactobacillus arabinosus* and *L. casei*. The growth promoting effect of anthranilic acid was not influenced by the simultaneous additions of much higher levels of orthanilic acid, orthanilamide, or 2-(o-anilamido)-pyridine.

Then Sevag and Green (1944, 1944b) began development of the concept of sulfonamide activity which is based upon the assumption that PABA is not an essential metabolite but rather a nontoxic analogue able to displace sulfonamides from an enzyme surface without itself inhibiting to the same extent the activity of the enzyme. Further, the concept associates sulfonamide action with the inhibition of respiratory enzymes, depriving cells of energy and so of the capacity for cell division and growth.

Sevag and Green (1944) reported that the sulfonamides inhibit the oxidative-reductive reactions leading to the synthesis of tryptophan by *Staphylococcus aureus*. These drugs did not directly interfere with the utilization of tryptophan. In other words, the addition of tryptophan to the medium abolished the inhibition of the sulfonamide by 50 per cent and completely stopped the bacteriostasis in the presence of pantothenic acid and riboflavin. In extending their work, these investigators (1944a) suggested that pantothenic acid mediates the metabolism of glucose leading to or involved in the synthesis of tryptophan and thereby causing bacterial growth.

A complete lack of any correlation between arylamine formation and sulfonamide resistance in *Staphylococcus aureus* cultures led to the conclusion that the development of resistance to sulfonamides is associated with the development of a sulfonamide resistant type of glucose metabolism (Sevag and Green, 1944b). The acquired resistance with respect to glucose metabolism was reversible and associated with amino acid metabolism through tryptophan. In this same series of reports, Sevag and Green (1944b) propose that pantothenic acid not only mediates the metabolism of glucose leading to or involved in the formation of tryptophan essential for the growth of the exacting strains of *Staphylococcus aureus* but also functions in the system which, in the presence of glucose, oxidizes tryptophan to arylamines. The critical action of the sulfonamide is stated by these investigators to be that of inhibition of tryptophan synthesis from glucose and amino acids.

Studying the details of the glucose metabolism blockage by sulfonamides, Sevag et al. (1945c) found that the point of inhibition is the oxidation of glucose to formic acid. These studies were carried out using *Escherichia coli*.

A more specific delineation of the point of action of the sulfonamides has been presented by Baumgärtel (1943) who states that PABA functions as a hydrogen transfer agent for *Escherichia coli* and other facultative anaerobes, enabling them to thrive under anaerobic conditions. Nicotinamide acts in the same way. Sulfonamides according to this hypothesis block hydrogen transfer. In the same vein, Jensen and Schmith (1942) suggest that these drugs displace PABA from an enzyme and are bound to the apoenzyme by means of the amino group. The sulfone groups function only in that they change the equilibrium constants of the dissociation of the apoenzyme-sulfonamide complex through an inductive action on the para-placed amino group.

Sevag et al. (1945, 1945a, 1945b, 1945c) were able to demonstrate blockage by sulfonamides of the bacterial decarboxylation of pyruvic acid. Sulfathiazole, most closely related to cocarboxylase, was the most powerful competitive antagonist. PABA reversed the sulfonamide effect on carboxylase activity and it is difficult to place the nutriline in the picture of substrates for the enzyme. In these same studies, Sevag and his associates established the relative specificity of PABA for sulfonamide inhibited reactions by proving its lack of effect in reversing the acetaldehyde inhibition of carboxylase.

Support of Sevag's concept of sulfonamide action has been presented by Altman (1946) who found a correlation between sulfonamide structure and the inhibition of respiratory enzymes. The inhibition of glucose-phosphate dehydrogenase by sulfonamides was reversed by triphosphopyridine nucleotide or substrate but not by PABA.

There are numerous observations difficult to integrate within the structure of the Sevag hypothesis. One of these is the series of studies demonstrating an absence of correlation between inhibition of growth and respiration (Wyss et al., 1942; Dorfman and Koser, 1942). Another complicating finding is that of increased synthesis of PABA by some sulfonamide resistant organisms (Landy et al., 1943; Landy and Gerstung, 1944). Finally, PABA has the capacity to antagonize sulfonamides over a wide range of concentrations and none of the respiratory coenzymes possesses a similar effect.

Recently, Sevag and his group have greatly extended their original studies on the mechanism of action of sulfonamides. In one paper (Steers and Sevag, 1949) they emphasize those studies which have indicated that sulfonamide resistant bacteria do not produce increased amounts of PABA (Goldsworthy et al., 1948) and offer as the logical alternative the concept of qualitative and quantitative metabolic differences between resistant and susceptible types with regard to amino acid utilization and synthesis. The primary site of drug action would then be the metabolism of glucose and amino acids. As an example, one resistant strain of *Staphylococcus aureus*

completely lost its ability to synthesize aspartic acid. The specific point of blockage is suggested as that of enzymes involved in oxidative deamination, flavoproteins with or without coenzyme linked dehydrogenase. Such blockage would prevent the cells from obtaining energy from amino acids or producing deaminated compounds as carbon sources for synthetic purposes. The organism can utilize glucose as an alternate method for obtaining α -keto acids and energy for the synthesis of amino acids.

Support of the Sevag concept of sulfonamide inhibition of dehydrogenases comes from the work of Gerundo (1950) who found inhibition of lactic dehydrogenase by succinylsulfathiazole. Glucose dehydrogenase was not affected. Bacteria capable of dehydrogenating succinic acid are resistant to sulfonamides but susceptible if this enzyme is lacking. *Shigella sonnei*, *Proteus vulgaris*, *Escherichia coli*, and *Aerobacter aerogenes* were capable of dehydrogenating succinic acid in the presence of sulfonamides.

Sevag and Steers (1950) have gone beyond the concept of inhibition by sulfonamides of glucose and amino acid metabolism to localize the mechanism at the point of glucose-glutamic acid relationship. They indicate that the transaminating role of glutamic acid in the synthesis of other amino acids would naturally place it at the center of the sphere of sulfonamide function in chemotherapeutic interference. Tryptophan also occupies a key position. Neither tryptophan nor glucose alone overcame the inhibition of sulfathiazole but in combination they were effective. Again, in this study, *Staphylococcus aureus* was the test organism.

Finally, this same group (Sevag et al., 1950a) from a study of sulfonamide resistant and susceptible strains of *Staphylococcus aureus* conclude that there is an interrelation of tryptophan and pyruvate metabolism. Resistant strains have a glucose or pyruvate metabolism which is resistant to the action of the drugs; they do not by-pass pyruvate metabolism. Most of the amino acids have been implicated in the modified metabolism associated with sulfonamide resistance, and in one case involving *Lactobacillus arabinosus* in which reversal of sulfonamide action did not occur with folic acid. Koft et al. (1950) have observed reversal with PABA and with some samples of lysine.

Mutant strains of *Neurospora crassa* have been obtained (Emerson, 1947) in which the antagonistic roles of PABA and the sulfonamides have been reversed. With these strains, the sulfonamides act as necessary factors for optimal growth and PABA is a potent fungistatic agent. Emerson emphasizes the probability that the sulfonamide actually is utilized by the *Neurospora* in its metabolism. The complexity of the metabolic concatenation of this mutant is reflected in its need for both sulfonamide and PABA for growth and by the fact that each can act as an antagonist for the other.

The observation of Emerson cannot be fitted into the pattern of sulfonamide interference with the synthesis of folic acid, but might be integrated

into the Sevag concept of sulfonamide blockage of glucose and amino acid metabolism. The structural similarity of PABA and the sulfonamides is so marked as to render probable the capacity of each to function as a cofactor or trigger mechanism for the same enzyme, assuming only slight modification in the reaction site structures.

Folic Acid Synthesis as the Site of Sulfonamide Action

It was only logical to anticipate elucidation of the site of action of sulfonamides with development of knowledge of the chemical nature of the natural forms of PABA. Auhagen (1943) had suggested that p-aminobenzoyl-L-glutamic acid was the answer. He claimed that this compound had ten times more activity than an equimolecular quantity of PABA as an antagonist of sulfanilamide. Williams (1944) failed to confirm his assertions.

In 1946, Lampen and Jones reported the noncompetitive antagonism between sulfonamides and folic acid. This antagonism occurred with *Streptococcus faecalis* R, *Lactobacillus casei*, *Lactobacillus arabinosus*, and a series of enterococci. It did not occur with *Escherichia coli*, *Staphylococcus aureus*, or *Diplococcus pneumoniae*; none of which requires either PABA or pteroylglutamic acid for growth. With those organisms manifesting protection by folic acid, thymine was also effective. They proposed that the synthesis of pteroylglutamic acid from p-aminobenzoic acid was the point of sulfonamide action. This would fit the pattern of competitive antagonism of sulfonamide and PABA and noncompetitive antagonism between sulfonamide and pteroylglutamic acid.

In general, sulfonamides are counteracted by folic acid when the test organisms themselves synthesize the vitamin; no antagonism occurs with those organisms requiring preformed folic acid for growth (Lampen and Jones, 1946a). Pteric acid antagonized sulfonamides with some folic synthesizing bacteria but not with others. The interpretation of this finding is that conversion of pteric acid to pteroylglutamic acid is sulfonamide sensitive in some of these organisms and not in others.

Emphasis has been placed by Lampen and Jones (1947) on inability of pteroylglutamic acid to replace PABA in growth promotion with some microorganisms, i.e., a mutant strain of *Escherichia coli* requiring PABA (Lampen et al., 1946). They offer in explanation of this observation the potentiality that permeability may be low in these cells to pteroylglutamic acid; that the cellular production of folic acid results in a molecular species differing slightly from the synthetic pteroylglutamic acid; or, finally, that PABA possesses some function beyond that of substrate for the formation of pteroylglutamic acid. Certainly, the question of permeability must play an important role in phenomena of this sort. Martin et al. (1949) have reported the complete inhibition of the in vivo antibacterial action of antibiotics and sulfonamides against streptococci by d-catechin. This antago-

nism is explained as being due to the inhibition by the d-catechin of hyaluronidase with resultant polymerization of hyaluronic acid and decreased cellular permeability. Inasmuch as it is a nonspecific phenomenon—effective against sulfonamides and antibiotics—the likelihood is that this proposal will be sustained.

The concept of sulfonamide action must account for the almost universal efficacy of PABA as an antagonist and for the limited ability of pteroylglutamic acid to function in this antagonistic capacity. It can be stated that the antisulfonamide effect of PABA has been found with all microorganisms tested excepting *Bacterium tularensis* (Tamura, 1944). At the same time, there are a number of microorganisms which are susceptible to the action of sulfonamides but with which pteroylglutamic acid does not antagonize the sulfonamide. Woods (1950) offers three possible explanations of this discrepancy in the basic concept. The points precisely parallel those offered by Lampen et al. (1946) in explanation of the inability of pteroylglutamic acid to replace PABA in the growth of some microorganisms. Again, it seems probable that permeability is the factor primarily responsible for the failure of pteroylglutamic acid to antagonize the action of sulfonamides on some bacteria. Inasmuch as a sulfonamide can be rendered ineffective against *Staphylococcus aureus* by virtue of altered permeability (Martin et al., 1949), it is not difficult to imagine a relative selectivity based upon a permeability which distinguishes between a molecule of the proportions of PABA and one of the magnitude of pteroylglutamic acid. In general, this factor of permeability forms a constant shadow for all studies of biological antagonism and must be given detailed consideration in the elaboration of any concept of mechanism.

While Lampen and Jones (1947) were studying the inability of folic acid to replace PABA in the nutrition of certain organisms, the English workers associated with Woods were investigating the ability of folic acid to reverse the growth inhibiting action of sulfonamides in those bacteria which require PABA. They found one series in which pteroylglutamic acid acted as a noncompetitive antagonist. With *Clostridium acetobutylicum* and *Lactobacillus plantarum*, the concentration of folic acid required for reversal of the sulfonamide was essentially the same as that required to replace PABA as a growth factor for these organisms; however, the molar concentration required for both purposes was from 10–100 times greater than that of PABA (Nimmo-Smith and Woods, 1948). They offer two alternatives in explanation of this finding: less efficient absorption of pteroylglutamic acid or lack of identity of this material with the form of this vitamin normally synthesized.

In an associated experiment, these same workers (Nimmo-Smith et al., 1948) were able to demonstrate a quantitative relationship between the concentration of PABA in a medium and the amounts of *Lactobacillus*

casei factor produced. The microorganism used was *Lactobacillus plantarum*. Another significant finding was that synthesis began at just that concentration of PABA normally found limiting for growth. The synthesis of the factor was inhibited competitively by sulfonamides. These findings strongly substantiate the concept of the existence of various forms of folic acid.

In addition to the series of bacteria with which pteroylglutamic acid acts as a noncompetitive inhibitor for sulfonamides, there is another group of PABA requiring microorganisms for which pteroylglutamic acid can be either a competitive inhibitor or entirely inactive.

Various Forms of Folic Acid

In explanation of the inability of pteroylglutamic acid to replace PABA in growth promotion with certain organisms, and of the failure of the glutamic acid derivative to reverse the action of sulfonamides, several groups of investigators (Lampen and Jones, 1947; Nimmo-Smith and Woods, 1948) raise the question of the nonidentity of various forms of folic acid. There are many reports favoring this position, and inasmuch as the concept seems the most logical explanation of various unexplained aspects of the action of sulfonamides, a review of the subject is in order.

Hall (1947, 1947a, 1947b) first proposed the existence of a multiplicity of folic acid factors in natural sources. His initial publication stated his belief that the folic acid of Mitchell et al. (1941) was not identical with pteroylglutamic acid but more nearly comparable to the vitamin B₉ of Binkley et al. (1943), although the close chemical relationship of the factors was conceded. Subsequently, he noted the reversal by both pteroylglutamic and folic acid of the inhibitor action of quinoxaline on the growth of *Streptococcus lactis* R (Hall, 1947a). In the last paper of the series, the ability of histidine to react with some component of a bacterial medium and thus form a product capable of functioning as folic acid was recorded along with the statement that this complex was neither folic acid nor pteroylglutamic acid.

Proof of the nonidentity of various folic acid factors came with the determination of the chemical nature of the *Streptococcus faecalis* R factor as formylpterioic acid (rhizopterine), which has greater growth factor activity than pterioic acid (Rickes et al., 1947). Again, formylpteroylglutamic acid reverses the growth inhibition of *Streptococcus faecalis* R by 7-methylpteroylglutamic acid more easily than does pteroylglutamic acid (Gordon et al., 1948).

Recently, Bond et al. (1949) by the application of inhibition analysis identified a factor differing from folic acid but closely related thereto which they named "folinic acid." While "folinic acid" seemed to account for the activity of hog liver extracts, they identified by chromatography two other

substances possessing activity in the *Lactobacillus casei* assay. Simultaneously, Bardos et al. (1949) disclosed their finding of enhanced potency "folinic acid" by thymidine and of the destruction of "folinic acid" by acid hydrolysis with resultant formation of a compound with all of the biological activities of folic acid.

Further proof of the multiplicity of folic acid factors lies in the fact that natural concentrates active for the growth of *Leucomastix citrovorum* reverse the toxicity of metabolite antagonists of folic acid under conditions which pteroylglutamic acid is ineffective (Broquist et al., 1950). Studies in mice also indicate that CF concentrates protected mice against the toxicity of 4-aminopteroylglutamic acid; pteroylglutamic acid was without effect. Later, this group (Broquist et al., 1950a) extended their work, reporting that thymidine reduced the requirement of CF factor of *Leucomastix citrovorum*. Furthermore, CF factor was a competitive inhibitor and thymidine a noncompetitive inhibitor of 4-aminopteroylglutamic acid. That CF factor is a biologically active derivative of pteroylglutamic acid seems certain, a compound possessing the activity of folic acid is liberated from it by acid hydrolysis. The identity of "folinic acid" and the CF factor seems probable.

While the implications of these findings are that folic acid may be an intermediate in the formation of the related molecules of similar functional activity, it is possible that some folic acid factors may prove to be more complex units in the biosynthesis of which folic acid is not required.

The secondary sulfonamide inhibitors of Kohn and Harris (1943) may also be fitted into the pattern of a theory of sulfonamide action. This has been done by Lampen and Jones (1947) and by Woods (1950) for amino acids and nucleic acid derivatives.

In the case of the nucleic acid derivatives, the evidence indicates that the formation of thymine and the purines occurs as a result of folic acid function. The secondary inhibitory characteristics of these factors would be due to their identity with the products of an enzymatic reaction. The antagonism should be and is noncompetitive. Stokes (1944) first suggested that pteroylglutamic acid acts as a cofactor in the synthesis of thymine. Later, Hitchcock et al. (1945) and Strandkov and Wyss (1946) observed that 5-bromouracil and similar compounds inhibit the growth of *Lactobacillus casei* with thymine but not with folic acid present in the medium. Lampen and Jones (1947) interpreted these findings to indicate that the product of the action of folic acid is a thymine precursor rather than thymine itself. As these investigators point out, such a mechanism would permit thymine promoting growth to be antagonized by agents ineffective against folic acid promoting growth.

Elucidation of these findings came with studies of folic acid antagonism. Shive et al. (1948) observed that methylpteroylglutamic acid was reversed not only by folic acid (Martin et al., 1947a) but also by thymidine.

thymine was not effective. The studies were conducted with *Leuconostoc mesenteroides*, an organism for which p-aminobenzoic acid is an essential growth factor.

It is probable that folic acid is involved in the synthesis not only of thymine but also of purines and pteridines. This contention is supported by the work of Rogers and Shive (1948) who found that the inhibitor-metabolite ratio was increased two to three times by the presence of purines in the medium and another ten times if thymine were also added. The folic acid analogue was not able to block some measure of growth with both thymine and purines present. The test organism for this work was *Lactobacillus casei*.

Folic acid deficiencies should not differ whether created by removing this factor from the medium, by blocking its formation by the use of a sulfonamide, or by displacing the formed folic acid molecule through the use of a compound such as methylfolic acid. This concept would necessarily be based upon the idea that the sulfonamides and the folic displacers possessed no action other than the specific one of interference with pteroylglutamic acid nutrition. It is difficult to understand therefore why thymine blocks sulfonamides but not folic acid antagonists, and conversely why thymidine reverses the folic displacers.

Generally, the interrelationship of thymine with the purines and the amino acids is so intimate as to make the study of one impossible without involvement of the others. These secondary antagonists of sulfonamide action are certainly products or the equivalent thereof of enzymatic reactions involving PABA. In this connection, Shive and Roberts (1946) proposed the term "product inhibition index," and defined it as the molar ratio of analogue to metabolite at which growth was limited by the rate of synthesis of the product. They found that for sulfanilamide, the methionine inhibition index was one-third that of the purine base inhibition index, and this in turn was one-tenth the inhibition index of the then undiscovered folic acid.

Three recent observations dovetail in support of the proposition that the prime action of sulfonamide is one of interference with folic acid synthesis and subsequent formation of purines and amino acids, the latter as secondary events to the prime phase of prevention of formation of folic acid. The first observation was the discovery by Stetten and Fox (1945) of the formation of an amine by *Escherichia coli* during sulfonamide bacteriostasis. The second was the identification of the amine as 4-amino-5-imidazolecarboxamide (Shive et al., 1947) and the recognition of the compound as a precursor in the biosynthesis of purines. The third and final observation was made by Woolley and Pringle (1950) when they found the same amine in cultures of *Escherichia coli* grown in the presence of 4-aminopteroylglutamic acid. Thus, whether the folic acid supply is limited by direct dis-

placement through 4-aminopteroylglutamic acid or by prevention of folic acid formation through sulfonamides, the same intermediary in the biosynthesis of purines accumulates in the culture. This can only be interpreted as indicating the blockage of purine synthesis at this stage due to inadequate supply of folic acid. The correlative of this is that the prime point of action of the sulfonamides is the displacement of PABA from an enzyme surface involved in the formation of pteroylglutamic acid. It will be recalled that *Escherichia coli* is an organism with which the inhibitory action of a sulfonamide is not reversed by PABA (Lampen and Jones, 1946).

In general, those substances known to antagonize noncompetitively the bacteriostatic power of the sulfonamides will also replace PABA as growth factors of bacteria. Secondary inhibitors for one bacterium will not necessarily function for another. The picture as a whole is complex. In addition to methionine, lysine, serine, glycine, allothreonine, threonine, leucine, valine, and possibly others, amino acids are known antisulfonamides. Among the nucleic acid derivatives not only thymine but also adenine, guanine, and xanthine function as sulfonamide antagonists.

Lampen et al. (1946) produced a mutant of *Escherichia coli* which required PABA for growth. With this mutant, they found that PABA could be largely replaced by adding thymine, purines, and amino acids. In the presence of these components, the bacteria were resistant to the action of sulfonamides. Parallel results have been reported (Woods, 1950) with a yeast, designated as "45," which grew in the absence of PABA if adenine and certain amino acids, specifically methionine and leucine, were supplied. While the adenine-amino acids combination did not completely reverse sulfonamide activity, a partial reversal ensued.

With microorganisms not requiring PABA, i.e., *Escherichia coli*, the amount of PABA needed to antagonize a sulfonamide is reduced markedly by the addition of methionine and still further upon the addition of guanine or xanthine (Shive and Roberts, 1946). Another such study (Winkler and deHaan, 1948) demonstrated similar reduction in the amount of PABA necessary for sulfonamide reversal upon the addition of methionine, xanthine, and serine. With pteroylglutamic acid or thymine added, no PABA was needed. Finally, the addition of valine brought the growth rate up to normal. Winkler and deHaan (1948) propose the existence of at least four enzyme systems involving PABA which can be blocked by sulfonamides and which are involved in the synthesis of methionine, xanthine, serine, and pteroylglutamic acid.

Comparatively few studies involving sulfonamides and folic acid analogues in the same test system have been made. One such study involving *Staphylococcus aureus* led to the observation that methylfolic acid is counteracted by pteroylglutamic acid, pteronic acid, sulfathiazole, and p-aminobenzoic acid, but not by p-aminobenzoyl-L-glutamic or glutamic

acids. In the same system, sulfathiazole was reversed by PABA and pterioic acid but not by pteroylglutamic, glutamic, or p-aminobenzoyl-L-glutamic acids (Martin et al., 1947, 1947a). From the standpoint of interpretation, two features of the results are confusing. First, why does pterioic acid reverse the sulfathiazole while pteroylglutamic acid does not? It may be that pterioic acid is the prime functional unit with this microorganism, or the factor of permeability may enter. Each displacing agent in itself may well affect permeability. In this case, the sulfonamide would have to reduce permeability and the folic acid analogue would either have no effect or tend to increase permeability. If the sulfonamide reduced permeability, the folic molecule might not pass into the interior of the cells. Second, what is the function of sulfathiazole in reversing the action of 7-methylfolic acid? Here, again, the explanation may be that the sulfonamide decreases permeability, rendering the cell immune to the analogue. This same basic mechanism would explain not only the sulfonamide effect in antagonizing 7-methylfolic acid but also the inability of pteroylglutamic acid to reverse sulfonamide action with this test system.

Both Auhagen (1948) and Möller et al. (1949) classify all bacteria into three groups. The first group (Gram-negative) do not require preformed folic acid for growth and are inhibited by sulfonamides. The sulfonamide effect is not reversed by added folic acid. The second group (Gram-positive microorganisms) do not require preformed folic acid, and are susceptible to the action of sulfonamides. With these bacteria, the growth inhibitor action of the drug is reversed by folic acid. Finally, the third group (Gram-positive) is designated as being constituted by those bacteria requiring preformed folic acid for growth and insensitive to the effect of the sulfonamides.

As an example of a Group I bacterium, they list *Escherichia coli* 1883 Co. With this microorganism, the effect of sulfathiazole was reversed by PABA and p-aminobenzoyl-L-glutamic acid but not by pterioic acid, ribopterin, folic acid, formylfolic acid, teropterin, or vitamin B₁₂. Thymine reversed about 50 per cent of the antagonism. Addition of purines, pyrimidines, amino acids, vitamins, etc., did not make the thymine effect complete. A strain of *Staphylococcus aureus* was used by these investigators as typical of Group II. All of the aforementioned compounds with the exception of vitamin B₁₂ reversed the action of sulfonamides on this group.

Möller et al. (1949) were able to transform Group II into Group III bacteria by four passages through a medium containing folic acid and sulfathiazole. The folic acid requirement was lost in two passages when the sulfonamide was omitted from the medium. Thymine and sulfathiazole brought about the same transformation as folic acid and the sulfa drug. This change could not be brought about through the use of a medium containing sulfathiazole and PABA. Group I strains could not be converted into Group III bacteria.

The basic mechanism of this transformation from bacteria not requiring folic acid to those dependent on the nutrilit is a fundamental observation. A metabolite analogue is used to block a normal synthetic mechanism; then the bacterium forgets for a time how to perform this synthesis and finally the capacity is reinstituted. Not only is this doubtless the explanation of the development of resistance or the loss of it but also it represents in bacteria a phenomenon which is the equivalent of a loss of memory and subsequent recovery.

Confirmation and extension of the work of Möller et al. (1949) was presented in 1950 by Harrison and Clapper who found that sulfonamide resistant strains of *Streptococcus mitis* had lost the ability to synthesize pteroylglutamic acid. Vitamin requirements of this sulfonamide resistant strain paralleled those of *Streptococcus faecalis* and were different from those of the parent sulfonamide susceptible types excepting in regard to biotin and pantothenate requirements. This report indicates major metabolic modifications with the development of sulfonamide resistance. These changes may reflect an extension of the effect of sulfathiazole beyond the sphere of pteroylglutamic acid metabolism or possibly suggest that loss of capacity for the synthesis of this nutrilit brought into play channels of biochemistry requiring modified vitamin needs.

Morgan (1948) first demonstrated the pteroylglutamic acid-PABA-sulfonamide interrelationship in the virus field. Using psittacosis virus as the test system, he found that not only PABA and folic acid but also pterioic acid reversed the growth-inhibitor effect of sulfadiazine (Morgan, 1948a). Both PABA and pterioic acid were competitive antagonists; pteroylglutamic acid was noncompetitive. Morgan's observations fit well into the pattern established for bacterial systems excepting in regard to the competitive antagonism manifested by pterioic acid. The implication of this finding is that pterioic acid is a stage intermediate between PABA and the final synthesis of folic acid by the psittacosis virus. This corresponds to the sulfonamide sensitive pterioic acid to pteroylglutamic acid sequence in bacterial systems (Lampen and Jones, 1946a).

Results similar to those of Morgan (1948, 1948a) were reported during the same year by Golub. He also used the psittacosis virus and found it susceptible to the action of sulfadiazine. PABA was a competitive antagonist. With the only other virus tested, lymphogranuloma venereum, the same antagonistic effect was not shown. Pteroylglutamic acid was required in higher amounts for reversal of sulfadiazine as contrasted to PABA. Resistant strains of psittacosis virus were developed to sulfadiazine and it was found that these same strains were also resistant to sulfathiazole and sulfamerazine.

Sevag and his co-workers have taken exception to some of the work of Lampen and Jones (1946a, 1947) in which they reported that PABA, p-

aminobenzoylglutamic acid, pteronic acid, pteroylglutamic acid, and pteroyl-triglutamic acid promote growth in cultures of *Lactobacillus arabinosus*, due to the conversion of the simpler factors into folic acid. Using this same test organism, Koft et al. (1950) found that the effect of these various PABA-containing units was due not to their integration into folic acid but rather to their disintegration into free PABA or p-aminobenzoylglutamic acid. Folic acid *per se* was not a growth stimulant for *Lactobacillus arabinosus*. Further, with this test organism folic acid did not reverse the growth inhibitory effects of sulfanilamide (Sevag et al., 1950). Any antagonism manifested by pteroylglutamic acid toward a sulfonamide is apparently due to decomposition of the vitamin with liberation of the simpler components which then counteract the drug.

Recapitulation

The cardinal feature of the mechanism of action of sulfonamides as bacteriostatic agents is their reversal by PABA. No other known chemical or group of chemicals completely possesses this power. Therefore, the premise can be made that all threads of any concept must be woven into the fabric of PABA metabolism. The theory may be promulgated that all secondary inhibitors are the products of enzymatic reactions associated with some PABA-containing folic acid molecule. Finally, a multiplicity of such factors must exist and the formation of some of these complex units may be via channels other than that of pteroylglutamic acid. Having stated the concept, let us attempt to fit the known facts into the pattern.

1. PABA is not solely an inhibitor of the action of sulfonamides as it functions as an antagonist for sodium p-nitrobenzoate, sodium phenylarsonate, etc. Evidence has not yet accumulated but the recorded observations could be fitted into the pattern of PABA displacement with resultant prevention of the formation of some folic acid type molecule.

In this connection, it is to be emphasized that the concept of biological relativity indicates a lack of absolute specificity in any system; and here we find PABA reversing the action of other antibacterial agents, and molecules similar to PABA in structure, such as 2-aminopyrimidine-5-carboxylic acid, displaying an antisulfonamide effect (Martin et al., 1944).

2. PABA will not counteract all of the effects of sulfonamides, e.g., production of fever and rash by sulfathiazole in patients. The concept as stated restricts its sphere to the bacteriostatic action of the sulfonamides. It is not likely that every manifestation of the toxicity of a sulfonamide will be mirrored in its inhibition of PABA complexes functioning in enzymatic systems. This is particularly so in view of the known power of the complex sulfonamides to inhibit enzymatic systems involving nicotinic acid or cocarboxylase. The correlative is also true, that in these selected enzyme systems nicotinic acid and cocarboxylase will reverse the inhibitory power of sul-

fapyridine and sulfathiazole respectively. In general, the pharmacological characteristics of sulfonamides are associated with the complex members of the series, and these effects are not reversed by PABA. As an example, sulfonamides inhibit the effect of nicotine on the isolated intestine and there is no reversal by PABA (Pick et al., 1944).

The correlation of acute toxicity values with complexity of the sulfonamide type indicates that the substituent units are primarily responsible or at least of major importance. Sulfanilamide is the least toxic; sulfathiazole, sulfadiazine, and sulfamerazine are roughly twice as toxic; sulfapyridine and sulfamethazine are three times as toxic, and sulfaguanidine about six times as toxic. Furthermore, the manifestations of toxicity vary from marked depression with sulfanilamide to extreme stimulation with sulfapyridine. It is safe to state that the toxic effects of these drugs do not indicate a similar pattern and it seems reasonable to assume that acute toxicities of sulfonamides are reflections of their antagonism of different enzymatic mechanisms.

Finally, while chronic toxicity studies in animals frequently have resulted in the precipitation of a folic acid deficiency state, this is due to inhibition of folic acid synthesis by bacteria in the gastrointestinal tract and it cannot in any manner be correlated with modification of PABA metabolism within the animal.

3. The proposed correlation of capacity for the synthesis of PABA and sulfonamide susceptibility fits well into the pattern of those microorganisms in which such relations exist; but there are bacteria (i.e., *E. coli*) resistant to the action of these drugs which do not produce increased amounts of PABA. If we assume that PABA is in some manner associated with the formation of all hypothetical types of folic acid molecules, it appears impossible to fit the reality into the hypothesis. This is particularly confusing because PABA will reverse the action of sulfonamides on *Escherichia coli*.

The means of integrating such observations into the concept follow two lines. One is that the method used for the determination of PABA may not have been specific, that is, it may not have revealed PABA in combination, or natural antagonists may have been present creating a false impression of the absence of increased PABA. The second line of thought is related to the idea that increased utilization rather than increased production of PABA might be the underlying principle. If the actual sulfonamide resistance is associated with the formation of some folic acid type molecule, then clearly there will be many factors other than PABA concentration which will control the extent of such reaction, i.e., enzyme concentrations, concentrations of other components of the folic molecule which would be substrates for the formative enzyme, absence of natural inhibitors, etc.

4. One major group of secondary inhibitors of the sulfonamides is the

amino acid series. These acids are noncompetitive inhibitors and behave in a manner suggesting that they represent the products of enzymatic actions associated with PABA metabolism.

The most probable mechanism of involvement of PABA-containing units in this system is that of interference by sulfonamides with transamination mechanisms. Sevag and Steers (1950) place glutamic acid and its relationship with glucose at the heart of their concept of inhibition of bacterial respiration by sulfonamides. They (Steers and Sevag, 1949) suggest as the specific point of blockage the enzymes involved in oxidative deamination, flavoproteins with coenzyme linked dehydrogenases. Many dehydrogenases require coenzymes I and II, and both of these cofactors are adenine-containing complexes.

If we now accept the explanation offered by Woods (1950) of Sevag's theory of sulfonamide action we arrive at a hypothetical but complete integration of secondary amino acid-sulfonamide antagonists within the framework of the general concept of PABA metabolism and sulfa activity.

An alternative has been proposed by Woods (1950) based upon reports (Gale, 1945; Giese and Tatum, 1946; Havinga et al., 1946) that sulfonamides interfere with peptide and protein synthesis. As Woods indicates, the decreased peptide synthesis could well be a manifestation of decreased amino acid formation. This latter suggestion would fit the experimental observations into the aforementioned concept.

5. The other major group of sulfonamide inhibitors of secondary nature is the nucleic acid derivatives. The evidence reviewed leaves little doubt that these compounds are products of enzymatic reactions involving a folic acid complex and consequently PABA. Two lines of evidence establish this point. First, thymine with certain purines can replace folic acid as a growth factor and as an antisulfonamide; and second, both sulfonamides and anti-folic compounds cause the accumulation of 4-amino-5-imidazolecarboxamide which is a precursor in the biosynthesis of purines.

6. The possibility that sulfonamides might prevent the enzymatic formation of PABA itself is immediately disposed of by the fact of the competitive relationship of the metabolite and inhibitor. If sulfonamides interfered with the enzymatic formation of PABA, the relationship would be non-competitive.

7. The failure of pteroylglutamic acid to replace PABA as a growth factor and as an antisulfonamide with some bacterial species is explained in accordance with multiplicity of folic acid complex types and permeability factors. The demonstration that the citrovorum factor is a biologically active derivative of pteroylglutamic acid (Broquist et al., 1950) establishes the first point. The second, the permeability factor, has been tentatively established by the reported ability of catechin to nullify the antibacterial action of sulfonamides (Martin et al., 1949) an observation which can only

be explained by the proposed inhibition of hyaluronidase by the bioflavonoid with resulting decreased permeability of the cell.

8. Reversal of the antagonistic roles of PABA and the sulfonamides is difficult to fit into the pattern of PABA displacement as the prime function of sulfonamides. If, as Emerson (1947) indicates, the sulfonamide actually is utilized in *Neurospora* metabolism, one would have to raise the possibility that an analogue of pteroylglutamic acid, in which PABA has been replaced by sulfonamide, might act as a replacer of the nutrilit. Evidence supporting the possibility of this contention is the observation that N-(4-((4-quinazoline)-amino)-benzoyl)glutamic acid, a folic acid molecule in which the entire pteridyl ring has been replaced, functions as a replacer for folic acid (Martin et al., 1947b). If a molecular modification of this magnitude can result in an active folic acid-like molecule, the above proposal is within the realm of possibility..

9. It is apparent that with those organisms requiring preformed folic acid for growth there would be no inhibition by sulfonamides unless the sulfonamides in some rare instance were to interfere competitively with the function of folic acid. Instances of competitive antagonism of sulfonamide and pteroylglutamic acid have been reported (Nimmo-Smith and Woods, 1948; Lampen et al., 1946). These could be due to degradation of pteroylglutamic acid to PABA with subsequent competitive antagonism of sulfonamide and PABA or direct competitive antagonism with pteroylglutamic acid itself. Many pteridine and purine molecules are known which antagonize pteroylglutamic acid and it is certainly not improbable that a sulfonamide would do so in a strictly competitive manner.

10. The role of methionine as a secondary inhibitor of sulfonamide action probably reflects the involvement of pteroylglutamic acid in transmethylation reactions. This proposal is based upon the demonstration by Dinning et al. (1949) that pteroylglutamic acid functions in the choline oxidase-enzyme systems as a constituent of a prosthetic grouping, and on the finding that choline oxidase activity is necessary for the utilization of the methyl groups of choline for transmethylation processes (Dubnoff, 1949).

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Chapter 4

AMINO ACID ANALOGUES, GENERAL

1. Mutual Antagonisms Manifested by Naturally Occurring Amino Acids.
2. The Antagonisms Manifested by Optical Antipodes.
3. Antipodal Specificity of Proteolytic Enzymes.
4. Miscellaneous Amino Acid Antagonists.
5. Recapitulation.

In presenting the metabolite analogues of amino acids, it is necessary to group a miscellaneous series of observations under a general heading, and then present as separate entities, wherever justified, the material on individual amino acids. To date, the body of work on methionine, phenylalanine, tyrosine, glutamic acid, aspartic acid, tryptophan, arginine, lysine, and histidine warrants treating each separately.

An attempt will be made to review briefly the specific functions of individual amino acids in the corresponding chapters on their metabolite analogues, but here the group functions of amino acids are considered. It is axiomatic that they form the building blocks of proteins. To this consideration, their role in the structures of physiologically important peptides must be added. These units containing but a few amino acid molecules must be reviewed both as the result of group functions of amino acids and as modified forms of single amino acids.

To comprehend the scope of proteins is as difficult as to grasp the significance of the universe. In the biological field, proteins are the universe. They are vital to every manifestation of life from the simplest virus to the most complex animal, the human being. A given organism produces structural configurations in proteins which are identical and, to the best of our knowledge, succeeding generations of any given species for ages have done the same thing. It has been estimated that there are 1600 different proteins in the body. For the consideration of metabolite analogues, it must be kept in mind that proteins are constantly in a dynamic state of amino acid transfer and are not static macromolecules. It is with this dynamic system that many amino acid displacers are primarily concerned; specifically, those which as their *in vivo* concentration increases first inhibit protein formation. Inhibition of any general process probably eliminates such compounds from a

practical role as chemotherapeutic or pharmacological agents, unless a differential requirement exists for specific tissues or parasites.

Among the roles carried out by proteins is that of *träger* or apoenzyme. For the purposes of displacement, this function is important. The obvious effect of an amino acid metabolite analogue would be prevention of the formation of protein; another would be displacement of a naturally occurring amino acid from the formed enzyme, preventing metabolic alteration of the nutritive. The coenzyme must be united with the protein component at some specific structural surface site, and it seems not unlikely that this surface site might be so modified by an amino acid displacer as to be unable subsequently to combine with the cofactor. Of course, metabolite analogues of the coenzyme would displace equally but by virtue of similarity to the prosthetic grouping rather than to the amino acids comprising the fixation point.

Mutual Antagonisms Manifested by Naturally Occurring Amino Acids

Imbalance of amino acid intake has long been known to produce toxic effects. Glycine (Riker and Gold, 1942), cystine (Curtis and Newburgh, 1924), lysine (Sullivan et al., 1932), tryptophan (Sullivan et al., 1932), tyrosine (Sullivan et al., 1932; Martin, 1943), serine (Fishman and Artom, 1942) and methionine (Earle et al., 1942) produce characteristic manifestations of toxicity when fed to animals under the proper experimental conditions. While Sullivan et al. (1932) had reported the mutual antagonism of tyrosine and cystine and of glycine, cystine, and glutamic acid, it remained for Medes et al. (1944) to interpret such effects as due to structural displacement mechanisms. They had observed the counteraction of the toxicity of serine in the rat by cysteine.

These mutually antagonistic phenomena were also found to occur when microorganisms were used as testing agents. Gladstone (1939) using *Bacillus anthracis* observed the interrelationships which existed between amino acids of similar chemical composition. Valine, leucine, and isoleucine, added singly to a mixture of amino acids which in itself supported growth (in the absence of the three amino acids), completely prevented growth. When they were added together, growth was actually improved and accelerated. The toxic effect of valine was counteracted by leucine, and vice versa; that of isoleucine could be neutralized only by the presence of both valine and leucine. Norleucine was similar to isoleucine in its action. Similar relationships were found between valine and threonine, valine and α -aminobutyric acid, and serine and threonine. The toxic effect of serine could also be neutralized by a combination of valine and leucine but not by either alone. There were in all these interrelationships definite and specific quantitative aspects.

Table I

MUTUAL ANTAGONISTS IN THE α -AMINO ACID SERIES

Toxic Agent	Antagonist	Test System	Reference
Valine	Leucine	<i>Bacillus anthracis</i>	Gladstone, 1939
Leucine	Valine	"	"
Isoleucine	Valine and leucine	"	"
Norleucine	Valine and leucine	"	"
Valine	Threonine	"	"
Threonine	Valine	"	"
Valine	α -Aminobutyric acid	"	"
α -Aminobutyric acid	Valine	"	"
Serine	Valine and leucine	"	"
Serine	Threonine	"	"
Threonine	Serine	"	"
β -Alanine	Asparagine	<i>Saccharomyces cerevisiae</i>	Nielsen and Hartelius, 1938
"	Aspartic acid	"	"
Arginine	Lysine	<i>Neurospora crassa</i> No. 4545	Doermann, 1944
Allothreonine	Methionine also other amino acids	<i>Proteus morganii</i>	Porter and Meyers, 1945
Norleucine	Methionine	" "	"
Norvaline	Methionine also other amino acids	" "	"
Glycine	Alanine	<i>Streptococcus faecalis</i>	Snell and Guirard, 1943
Threonine	"	"	"
Serine	"	"	"
β -Alanine	"	"	"
Norleucine or norvaline	Methionine	<i>Escherichia coli</i>	Harris and Kohn, 1941
Aspartic acid	Glutamine, glutamic acid, or asparagine	<i>Lactobacillus casei</i>	Feeney and Strong, 1942
Asparagine	β -Alanine	Yeast	Sarett and Cheldelin, 1945
Leucine	Isoleucine and valine	<i>Pasteurella pestis</i>	Doudoroff, 1943
Phenylalanine, norleucine, or norvaline	Isoleucine and valine	<i>Neurospora crassa</i>	Bonner, Tatum and Beadle, 1943
Threonine	Serine	<i>Lactobacillus delbrueckii</i> <i>L. casei</i> <i>Leuconostoc mesenteroides</i> <i>Streptococcus faecalis</i>	Meinke and Holland, 1948

Table I—(Continued)

Toxic Agent	Antagonist	Test System	Reference
Leucine, valine, or methionine	Isoleucine	<i>Lactobacillus arabinosus</i>	Brickson et al., 1948
Isoleucine	Leucine	"	" "
Aspartic acid or asparagine	Glutamic acid	"	" "
Norleucine	Methionine	<i>Escherichia coli</i> <i>Lactobacillus pentosus</i>	Harding and Shive, 1948
"	"	<i>Escherichia coli</i>	Lampen and Jones, 1947
Glutamine	Glutamic acid	<i>Staphylococcus aureus</i>	Grossowicz, 1948
Tryptophan	Phenylalanine	<i>Streptococcus faecalis</i> R	Beerstecher and Shive, 1947
Tyrosine	Phenylalanine	<i>Escherichia coli</i>	Beerstecher and Shive, 1947a

In the interests of brevity, the results of amino acid antagonism studies are presented in Table I. In general, the interrelationships are between structurally similar compounds, e.g., arginine and lysine, tyrosine and phenylalanine, valine and leucine, etc., but there is one recorded instance of an aromatic amino acid, phenylalanine, exerting a toxic action reversed by valine and isoleucine (Bonner et al., 1943). This result with *Neurospora* is paralleled to some extent by in vivo studies, using rats, in which

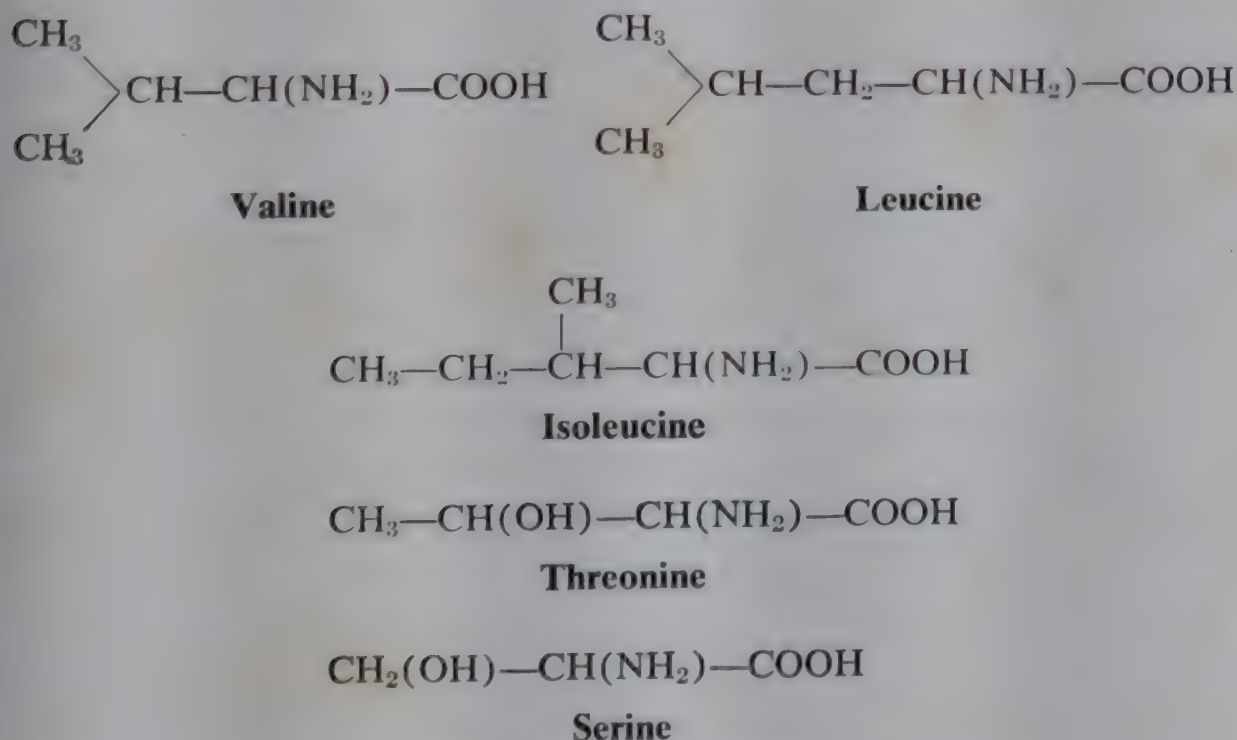


FIG. 1. Amino acid structures.

both glycine and cystine tended to reduce the toxicity of tyrosine (Marr 1947). It is probable that all mutual antagonisms exhibited by amino acids will be affected by vitamin and possibly even mineral balance. The etiology of many disease states may be associated with the imbalance. Medicine has for too long considered only the negative and the positive, deficiency disease as a reflection of absence of a given factor and bacterial and viral diseases as positive in the sense of the presence of a given infectious agent. To this limited concept must be added not only the double or multiple negative but also the negative-positive etiology of some disease states. As an example, tyrosine is known to produce a hypertensive state if fed in excess to rats; riboflavin deficiency sensitizes the rat to this toxic action; cystine tends to counteract the deleterious effect of tyrosine. It is possible therefore that the precipitating mechanism in some types of hypertension may be the simultaneous existence of three nutritional abnormalities—excessive tyrosine intake, and riboflavin and cystine deficiencies. As one of these will not produce the syndrome associated with all three.

The Antagonisms Manifested by Optical Antipodes

The inhibitory action of optical antipodes was reported in 1944 by Fieser et al. The unnatural forms of valine and leucine retarded the growth of *Lactobacillus arabinosus*. Later, the same investigators (Kobayashi et al. 1948) found that these isomers were also toxic to *Escherichia coli*. D-Alanine was not inhibitory for *Lactobacillus arabinosus* but was active against the growth of *Escherichia coli*. The authors indicate the correlation between the growth inhibitory capacity of such agents and the hydrolyzability of peptides containing unnatural amino acids, when subjected to the action of peptidases. Optical specificity of proteolytic enzymes is considered in the next section.

Lyman and Kuiken (1948) discovered that the D-isomer of isoleucine was utilized by *Lactobacillus arabinosus* in the presence of pyridoxamine. Leucine tended to render the optical antipode nonavailable to the organism.

In animals, it may well be that the D-forms are less toxic than the L-isomers; for example, D-methionine produced less growth retardation in rats than the L-form at corresponding levels (Van Pilsum and Berg, 1950).

Tyrocidine and gramicidin are high molecular weight polypeptides containing D-amino acids. Whether or not the antibiotic activity of these agents is specifically related to the presence of the unnatural forms of amino acids has not been established. Lipmann et al. (1941) have suggested that the presence of D-amino acids in gramicidin may correlate with its antibacterial properties. Work (1948), on the other hand, theorizes that antibiotics containing the unnatural forms of amino acids are active not because they contain these antipodes but rather due to their individual structures, in which the D-amino acid is an integral part.

The fact that peptides containing D-leucine fail to exhibit as much activity as the free amino acid (Fox et al., 1948) indicates that the viewpoint of Work (1948) reflects the true situation and that the activity of an antibiotic such as gramicidin is not even partially represented by continuous D-amino acid residues.

Table II
ANTAGONISM BY OPTICAL ANTIPODES

Enzyme Agent	Antagonist	Test System	Reference
Leucine	L-Leucine	<i>Lactobacillus arabinosus</i> — <i>Escherichia coli</i>	Fox et al., 1944; Kobayashi et al., 1948
Isoleucine	L-Isoleucine	<i>Lactobacillus arabinosus</i>	Lyman et al., 1948
Valine	L-Valine	<i>Lactobacillus arabinosus</i> — <i>Escherichia coli</i>	Fling and Fox, 1945; Kobayashi et al., 1948
Alanine	L-Alanine	<i>Escherichia coli</i>	Kobayashi et al., 1948
Alanine	L-Alanine	<i>Aerobius</i> spores	Hillis, 1948
Histidine	L-Histidine	Histidase	Arson and Murky, 1934

Antipodal Specificity of Proteolytic Enzymes

Optical specificity is shown by some proteases and peptidases (Bergmann and Fruton, 1941; Stahmann et al., 1946). Peptides containing D-amino acids in certain positions are not acted upon by these enzymes. Only those peptide linkages are split which have as one component either the L-form or an asymmetric amino acid or glycine. In general, the peptidases which do act on substrates containing the unnatural forms of amino acids, do so at a slower rate. Carboxypeptidase is unable to hydrolyze synthetic peptides in which the terminal amino acids are of the D-configuration (Stahmann et al., 1946).

While substrate specificity is a point of great interest in the general consideration of displacement, it is with inhibition of proteolytic enzymes that we are primarily concerned. Extensive investigations have been made on inhibitors of two pancreatic proteases, α -chymotrypsin and carboxypeptidase.

Neurath and Schwert (1950) have established three structural requirements of active substrates for chymotrypsin. They are: an aromatic ring, or condensed aromatic rings, removed by one or more methylene groups from the α -carbon atom; an uncharged polar group with proton donation capacity, substituted on the α -carbon in the L-configuration—e.g., a benzoyl or nicotinyl derivative of an α -amino group; and a hydrolyzable bond, e.g., amide, hydroxamide, hydrazide, peptide, or ester.

Kaufmann and Neurath (1949, 1949a) studied a series of structural analogues of specific substrates of chymotrypsin and found that the pres-

Table III

INHIBITORS OF CHYMOTRYPSIN*

	Structure	Configu- ration	K_i 10 ^{-2M}
1.	$\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{COOH}$		0.45
2.	$\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{NH}_2$		
3.	$\text{C}_6\text{H}_5\text{CH}_2\text{CHCOOH}$ NHCOC_6H_5	D	1.5
4.	$\text{C}_6\text{H}_5\text{CH}_2\text{CHCOOH}$ NHCOC_6H_5	L	2.9
5.	$\text{C}_6\text{H}_5\text{CH}_2\text{CHCOOH}$ NHCOC_6H_5	DL	2.6
6.	$\text{C}_6\text{H}_5\text{CH}_2\text{CHCOCH}_3$ NHCOCH_3	DL	0.79
7.	$p\text{-HOC}_6\text{H}_4\text{CH}_2\text{CHCOCH}_3$ NHCOCH_3	DL	0.72
8.	$p\text{-CH}_3\text{COOC}_6\text{H}_4\text{CH}_2\text{CHCOOH}$ NHCOCH_3	L	2.0
9.	$\text{C}_6\text{H}_5\text{CH}=\text{CCOOH}$ NHCOCH_3		1.8
10.	$\text{C}_6\text{H}_5\text{CHCOOH}$ NHCOC_6H_5	DL	0.78
11.	CH_2COOH NHCOC_6H_5		4.1
12.	$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CHCOOH}$ NHCOC_6H_5	DL	4.6
13.	$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CHCOOH}$ NHCOCH_3	DL	Very large
Related structures which are not inhibitors			
14.	$\text{C}_6\text{H}_5\text{CH}_2\text{CHCOOH}$ NH_2	D,L	
15.	$\text{C}_6\text{H}_5\text{CH}_2\text{CHCOOH}$ COOH	dl	
16.	CH_2COOH NHCOCH_3		

* From Neurath and Schwert, *Chem. Rev.*, **46**, 69, 1950. By permission of The Williams and Wilkins Co., Baltimore, Md.

ence of the "susceptible bond" was not essential for inhibitory action. It could be absent or replaced by a ketone group. The most active inhibitors were benzoyl-DL-phenylalanine and DL-1-phenyl-2-acetaminobutanone-3. The displacement effect was of the competitive type. Table III taken from Neurath and Schwert (1950) lists the entire series studied.

K_i is the dissociation constant of the enzyme-inhibitor complex. From these values, conclusions were drawn concerning the structural requirements related to those for substrates. The first of these is the amino acid side chain which for substrate concerns an aromatic ring and for inhibitor is of comparatively minor importance. There is however a certain correlation between aromatic and aliphatic side chains and affinity for substrate or inhibitor which led these investigators to assume that the side chains are oriented in the same way on the enzyme surface.

The second structural requirement for substrate was that of an uncharged polar group with proton donation capacity. In the inhibitor series, this group could be a hydrogen atom, a benzamido group, or an acetamido group but not a carboxyl or amino group if an α -carboxyl was already present. From their data, these investigators conclude that in general benzoylated aromatic amino acids should be potent inhibitors of chymotrypsin, but point out that the most potent inhibitor known, β -phenylpropionic acid, is not in this group. Stereospecificity of inhibitors was observed; generally, the D-form was the more powerful. In addition to the compounds studied by Kaufmann and Neurath (1949, 1949a), the D-form of N-acetyltyrosylglycinamide has been found to inhibit competitively chymotrypsin (MacAllister et al., 1949).

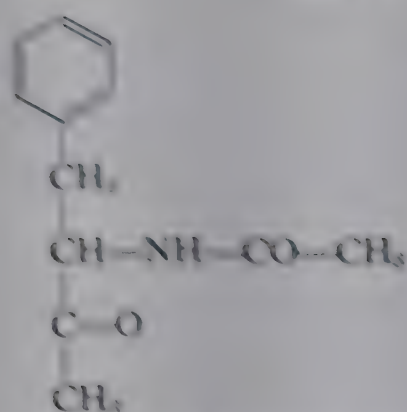
The third structural requirement for substrate was the hydrolyzable bond and as pointed out above this is not a prerequisite for inhibitor potency.

With carboxypeptidase, the structural substrate requirements are similar to those for chymotrypsin, namely, an aromatic amino acid of the L-series, containing a free α -carboxyl, with its amino group linked by a peptide bond to a monoaminomonocarboxylic acid, the amino group of which is masked by an acyl group (Neurath and Schwert, 1950).

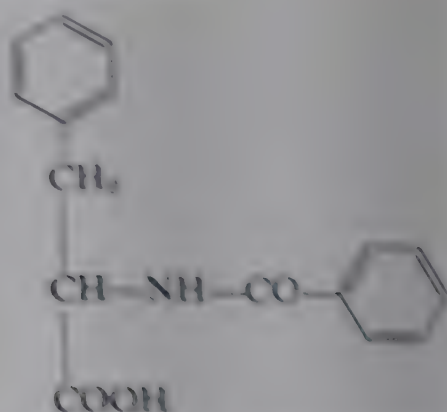
Inhibitors of carboxypeptidase are considered in four categories: (1) reaction products of substrates, (2) substrates, (3) structural analogues of substrates, and (4) inorganic ions.

Two examples of inhibition by reaction products are given by Neurath and Schwert (1950): chloroacetate inhibits noncompetitively the hydrolysis of the chloracetyl derivatives of tyrosine, phenylalanine, and β -phenyllactic acid and also of carbobenzoxyglycylphenylalanine; and L-phenylalanine inhibits competitively the hydrolysis of carbobenzoxyglycyl-L-phenylalanine.

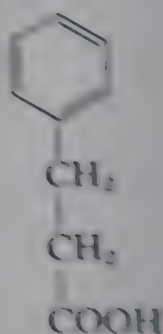
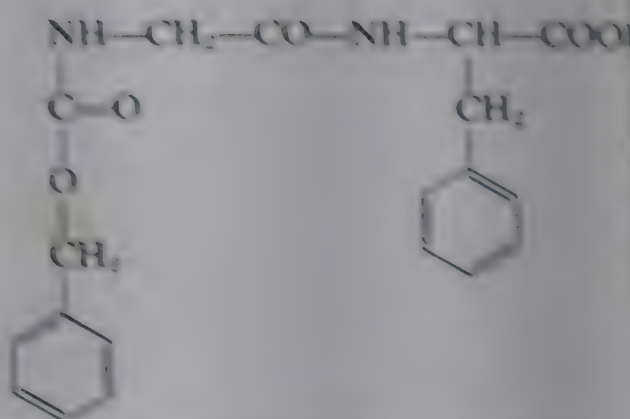
Inhibition of the action of carboxypeptidase on a highly susceptible by a less sensitive substrate remains questionable and will not be considered here.



DL-1-Phenyl-2-acetaminobutanone-3



Benzoyl-DL-phenylalanine

 β -Phenylpropionic acid
(hydrocinnamic acid)

Carbobenzoxycylphenylalanine

FIG. 2. Inhibitors of chymotrypsin or carboxypeptidase.

The action of structural analogues of specific substrates as inhibitors is presented in detail by Elkins-Kaufman and Neurath (1949). They found the following compounds inactive: N-methyl-DL-phenylalanine, dimethyl-DL-phenylalanine, DL- ω -phenylethylamine, β -phenylethylhippuric acid, carbobenzoxyglycine, and carbobenzoxyglycylglycine. A series of active inhibitors and their corresponding K_i values are: D-phenylalanine, 2.0; D-histidine, 20.0; hydrocinnamic acid, 0.062; p-nitrophenylacetic acid, 2.5; phenylacetic acid, 0.39; and β -phenylbutyric acid. While D-phenylalanine was the most powerful, the D-forms of alanine, leucine, lysine, and histidine were also active (Elkins-Kaufman and Neurath, 1949). The inhibitory activities of the unnatural forms of the amino acids paralleled roughly the ease of hydrolysis of their corresponding peptide by carboxypeptidase.

These investigators (Elkins-Kaufman and Neurath, 1949) draw conclusions from their results relative to the structural requirements for inhibitors: the α -amino group of D-phenylalanine is not required, as hydrocinnamic acid is more powerful; the distance of separation of the p

from the carboxyl group is a determinant for potency; the phenyl ring can be replaced; there must be a free carboxyl.

The fourth category of carboxypeptidase inhibitors is that of inorganic compounds which remains clouded by conflicting reports and will not therefore be discussed.

A novel approach to the study of the specificity of carboxypeptidase is that of Dunn and Dittmer (1950) who prepared peptides containing known potent antagonists of amino acids. The compounds prepared were: carboxyglycyl- β -2-thienylalanine, carboxybenzoylglycyl- β -1-naphthylalanine, carboxybenzoylglycyl- β -2-naphthylalanine, and carboxybenzoylglycyl- β -thiophenylalanine. As substrates, the naphthyl analogues were resistant to the action of carboxypeptidase, the thienyl compound was hydrolyzed with about one-half the speed of its benzene relative, and the thiophenyl compound was the equivalent of carboxybenzoylglycyl- β -thiophenylalanine. The β -2-naphthylalanine compound was the only one of the three tested as an inhibitor of enzymatic activity and it was found to be weakly active, delaying the rate of hydrolysis to a moderate degree.

Miscellaneous Amino Acid Antagonists

Mullwain (1941) observed the inhibitory action of α -aminosulfonic acids on the growth of *Proteus* and *Staphylococcus* in a chemically defined medium. The action was reversed by the addition of α -aminocarboxylic acids, which did not need to correspond closely to the structure of the inhibiting aminosulfonic acid. The antagonism was specific only in the sense that it was confined to the α -amino acids.

When staphylococci were made independent of meat added amino acids by long training, the aminosulfonic acids were no longer inhibitory. These amino acid metabolites were inhibitory in concentrations ranging from 10^{-5} to 10^{-2} molar when *Proteus* was the test organism. A relative specificity was observed, as illustrated by the greater capacity of valine to reverse α -aminocetanesulfonic acid, compared to that of glycine and alanine. The valine action was complete; that of the other two amino acids was only partial. Another manifestation of partial specificity was observed by Mullwain (1941) in the failure of alanine at M/100 to reverse α -aminocetanesulfonic acid, whereas other amino acids reduced the inhibition at M/2000. Glycine was particularly effective. α -Aminophenylmethanesulfonic acid was reversed by glycine and phenylalanine but not by valine and β -alanine.

The seeming lack of specific structural relationships between sulfonic and carboxylic amino acids is in accord with general concepts of interference occurring at enzyme surfaces. Proteolytic enzymes are not specific to particular amino acids, and though differences in speed of enzyme attack on peptides of different amino acid compositions do occur, the same differences are seen in the relative specificities of the sulfonic acid antimetab-

olites and the natural amino acids. Through the extension of his studies of *Escherichia coli*, *Proteus vulgaris*, *Bacterium typhosum*, and *Corynebacterium diphtheria*, McIlwain (1941) found that the aminocarboxylic acid which best reduced the inhibition of a given aminosulfonic acid varied with the organism, a fact which he explained by assuming different synthetic abilities and needs of the organisms.

Spizizen (1943) found that the sulfonic acid analogue of glycine (aminomethanesulfonic acid) inhibited bacteriophage multiplication on *Escherichia coli* which was stimulated by α -amino acids, particularly glycine and glycine anhydride. Studying the effect of metabolite analogues on the growth of vaccinia virus in chick embryonic tissues, Thompson (1947) found α -aminomethanesulfonic acid, α -aminoisobutanesulfonic acid, and α -aminophenylmethane sulfonic acid inhibitory in concentration of 1×10^{-3} . Four sulfonic acid analogues—aminomethanesulfonic acid, α -aminoethanesulfonic acid, α -aminoisobutanesulfonic acid, and α -amino- β -phenylethanesulfonic acid—were used by Greenberg and Schulman (1947) in an attempt to inhibit the growth of a sarcoma in mice. Under the conditions of the experiments, the final growth of the tumor was not appreciably affected.

A new member of the aminosulfonic acid series, 1,1-aminopropanesulfonic acid, which is the sulfonic acid analogue of α -aminobutyric acid, was prepared and tested by Mendel and Visser (1950). The compound inhibited irreversibly the growth of *Lactobacillus casei*.

Several molecules of the vinylene amino acid type have been prepared and tested as antibacterials. Allylglycine, methallylglycine, and crotylglycine (Dittmer et al., 1948) inhibited the growth of *Escherichia coli* and *Saccharomyces cerevisiae*. Despite their structural similarity to cysteine, no statement has been made on nutritive reversal of these toxic agents. The activity of allylglycine led Gershon et al. (1949) to prepare the corresponding acetylenic, propargylglycine, which proved more active against yeast than against *Escherichia coli*.

β -Methyl- α -ketovaleric acid which is the keto derivative of isoleucine, exhibits a tendency to block the conversion of the keto acid analogue of valine to valine (Bonner, 1946). Another possible valine antagonist, β -chloro- α -aminobutyric acid, was prepared by Klooster et al. (1948) and found to inhibit the growth of *Saccharomyces cerevisiae*, an action reversed by valine and leucine.

An extensive study of the effect of N-substituted amino acid analogues on the growth of *Lactobacillus* was reported by Harper et al. (1949). In general no effect was observed, but there were two exceptions. N-Phenylleucine was inhibitory to the growth of *Lactobacillus arabinosus*; reversal was complete with leucine. N-Phenylphenylalanine displaced phenylalanine tested on *Leuconostoc mesenteroides*.

Recapitulation

Numerous examples of the antagonism of one amino acid for the utilization of another are given in Table I. Viewed in its broadest implications, the metabolite analogues probably form the foundation of metabolic stability as designed by nature. Consider the synthesis of proteins in the animal organism. These complex units are constant in composition, and one factor in that constancy may well be mutual antagonisms between naturally occurring amino acids. The enzymes forming proteins actually have a constant milieu of available amino acids presented to them; not because the medium itself is constant in amino acid composition but because the interrelated antagonistic phenomena "buffer" the system. If the imbalance becomes too great, toxicity supervenes. Nature by this procedure stabilizes the amino acid supply not in an absolute quantitative sense but in a relative quantitative sense.

This delicate balancing mechanism may well apply equally to the control of the metabolism of the individual amino acids. For example, competitive antagonisms for enzyme site might prevent excessive formation of histamine if excess histidine were supplied to a decarboxylase. A phenomenon such as is seen in the inhibition of histidine decarboxylase by dihydroxyphenylalanine illustrates the case.

Competitive, and possibly noncompetitive, metabolite antagonisms may form the very foundation of life by stabilizing available relative nutritive concentrations. This is the "relativity theory" of the biological world.

In view of this concept it becomes important to consider metabolite antagonists in two categories, namely, natural and synthetic, restricting the term synthetic to those antimetabolites not found in nature or even more specifically to those antagonists not normally occurring in the system under study. The class of natural antagonists becomes of great importance in physiology; the synthetic class in pharmacology and chemotherapy. This is a general rule but it does not exclude practical applications of natural antagonists.

An important practical consideration centering around natural amino acid antagonisms lies in the complications this phenomenon presents to microbiological assay technics. The amino acid composition of the medium must be properly balanced. Consideration must be given to balance not only of amino acids, but of vitamins and minerals as well; and finally to the proper concentration relations of these factors to one another. The known limitations of accuracy of microbiological technics doubtless find explanations in the relative available concentration of metabolites presented to the test organism. Logically, the design of basic media should provide proper balance but in moderately high absolute concentrations. Such precaution should minimize the effect of addition of extraneous inhibitor metabolites along with the nutrient to be bioassayed.

The importance of studies on substrates and inhibitors of proteolytic or proteosynthetic enzymes is emphasized by the recent highly significant observations of Simmonds and Fruton (1950) in which they found that the bacteriostatic action of penicillin against at least one specific microorganism was due to inhibition of peptide synthesis. The test bacterium required L-leucylglycine for optimal growth but it did grow in the presence of equimolar concentrations of L-leucine and glycine. Penicillin exerted a slight inhibitory action in the presence of the peptide but in the presence of the amino acid its effect was marked. Using individual amino acids, these investigators observed a relatively greater effect against glycine than against L-leucine. A consideration of this report taken in conjunction with that of Gale and Taylor (1946, 1947) demonstrating that penicillin inhibited the utilization of glutamic acid by *Staphylococcus aureus*, and that of Simmonds and Fruton (1949) showing the bacteriostatic effect of glycyl-L-leucine on *Escherichia coli* as a function of leucine displacement, leads to the conviction that through this approach new antibacterial agents will be discovered.

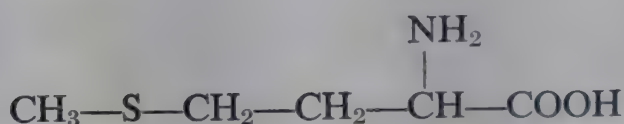
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Chapter 5

METHIONINE ANALOGUES



Methionine
 γ -Methylmercapto- α -aminobutyric acid

1. Metabolite Analogues Produced by Modification of the Methylmercapto Radical.
2. Metabolite Analogues Produced by Molecular Modifications Other than as in Section #1. Also, Miscellaneous Inhibitors.
3. Methionine and the Sulfonamides.
4. Enzymatic Aspects.
5. Recapitulation.

Methionine, an essential amino acid, plays a vital role in transmethylation, which as the name suggests involves intermolecular transfer of methyl groups. It is replaceable in the diet of the rat by a combination of homocystine and either choline or betaine. Transfer of the labile methyl radical of methionine to choline or creatine has been demonstrated by using deuterium as a tag. It seems probable that cleavage of the carbon to sulfur bond involves phosphate bond transfer and adenosinetriphosphate. Fig. 1 presents a summary of the metabolic transformations of methionine.

Cystine reduces but will not replace methionine requirements. As a result of its transmethylation power, methionine brings about the *in vivo* synthesis of choline and therefore possesses a lipotropic activity which is reflected in capacity to stimulate phospholipid formation. It thus functions indirectly in fatty acid transport and prevents the fatty livers which otherwise develop in animals on high fat diets.

Both optical antipodes of methionine are effective in the rat and probably in man. It is to be recalled that the lipotropic power of choline is not based on its transmethylation capacity. In other words, methionine acts only by furnishing methyl radical for the synthesis of choline, which acts by virtue of its phospholipid forming capacity. Substances such as guanidoacetic acid, nicotinamide and cystine, or any similar material which draws off labile

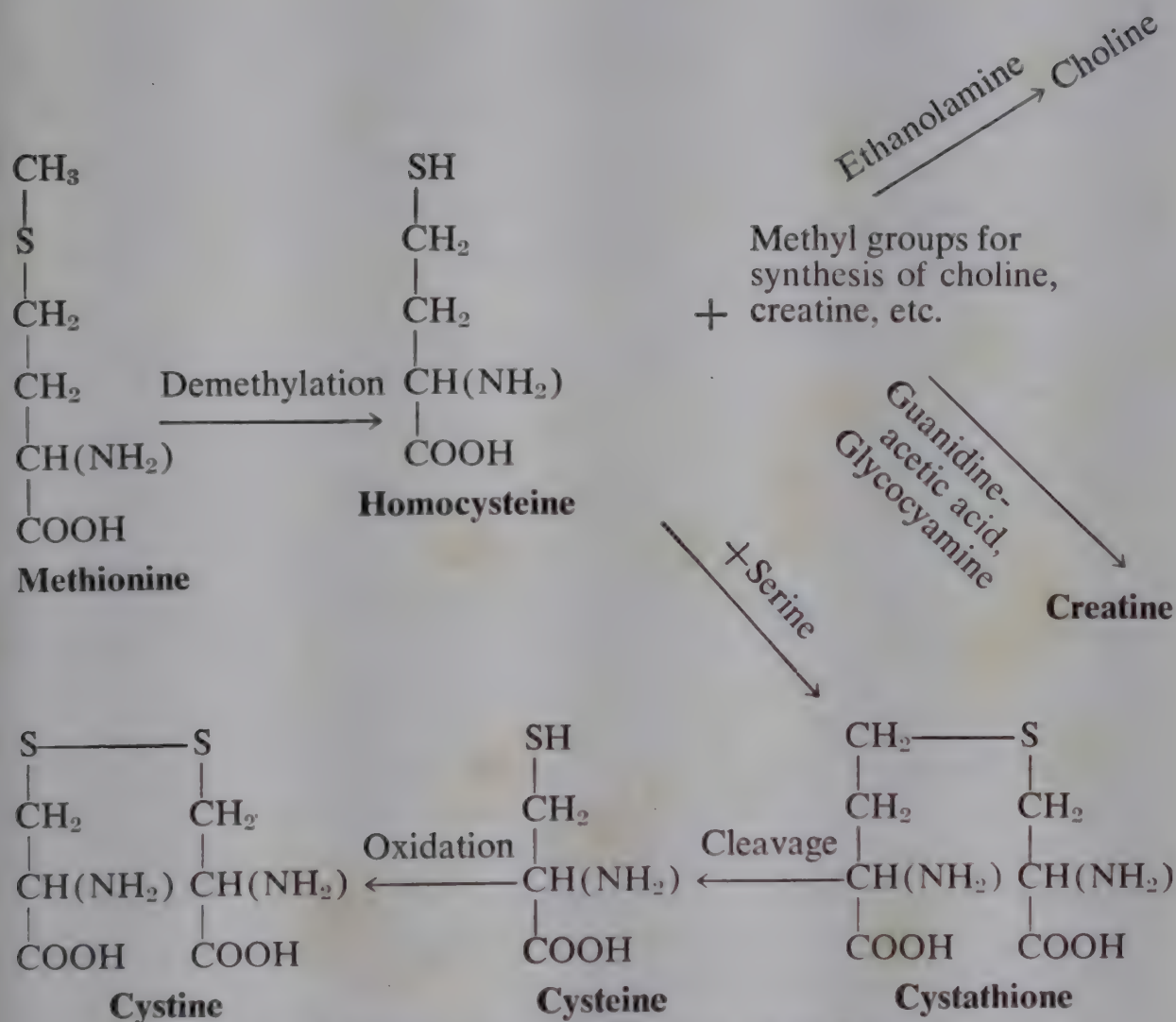


FIG. 1. Metabolic transformation of methionine.

methyl may in a sense be regarded as antagonistic to the methylating function of methionine. For example, the transmethylation processes required for the formation of trigonelline and N-methylnicotinamide drain the supply of essential methyl. The importance of methionine to creatine synthesis *in vivo* is greater viewed in the light of the fact that phosphocreatine is the immediate energy source in muscular contraction.

The essentiality of methionine is not associated solely with its labile methyl but also with its ability to supply sulfhydryl. Cystine or methionine is essential to the normal metabolism of aromatic amino acids such as phenylalanine and tyrosine, and these amino acids are keys to many detoxication mechanisms.

The unnatural form of methionine undergoes deamination and the keto form is then transaminated with resultant inversion to the natural form of the acid. The oxidative metabolism of methionine proceeds to the α -keto acid and possibly to the α -hydroxy acid but beyond this little is known of its fate except that it appears in the urine in the form of taurine, mercapturic acid, and inorganic sulfate.

The metabolism of methionine is so closely related to that of choline and cystine that it is difficult to separate the pathological manifestations; however, it appears that methionine deficiency causes a defective plasma protein and hemoglobin production. If there is a concomitant cystine deficiency liver necrosis and hair changes occur. If a choline deficiency is superimposed then fatty livers and hemorrhagic kidneys develop. Absence of methionine from the diet of experimental animals results in loss of hair, cessation of growth, and death.

Lactobacillus arabinosus and *L. casei* require methionine and can be used in a microbiological assay for this amino acid. In addition to these, other bacteria such as *Phytobacterium phosphoreum*, *Corynebacterium diphtheriae*, and *Clostridium sporogenes* will not grow unless methionine is present in the medium as an accessory growth factor. *Eberthella typhosa*, *Streptococcus viridans*, *Diplococcus pneumoniae*, *Shigella dysenteriae*, and *Neisseria gonorrhoeae* probably require the amino acid for optimal growth. Some microorganisms, *Neurospora crassa* for one, synthesize p-aminobenzoic acid from methionine; either compound will support growth.

Methionine has been used clinically in the treatment of toxemia and hemolytic disease of the newborn, in the hepatorenal syndrome of post-delivery shock, eclampsia, toxic purpura, chronic hepatitis, liver damage in general, in promotion of wound healing, in alcoholic cirrhosis of the liver, fatty livers, hepatic cirrhosis, toxic hepatitis, infectious hepatitis, and hepatic necrosis. Final evaluation of these therapeutic procedures awaits extension of clinical research in the field.

As a model for the design of metabolite analogues, the methionine structure immediately suggests modifications in the methylmercapto radical, which is its characterizing unit. It would seem probable that other structural changes might well lead to lack of specificity. Sulfonic acid analogues would almost surely displace amino acids more or less indiscriminately. Amino-nitrogen substituted units such as an N-methyl would be metabolized in the same manner as the parent amino acid.

Metabolite Analogues Produced by Modification of the Methylmercapto Radical

In 1941, Harris and Kohn studied the action of ethionine as a displacer of methionine. From their observations particularly as reflected in the failure of p-aminobenzoic acid to reverse the activity of ethionine, they concluded that p-aminobenzoic acid was related to methionine through some metabolic pathway. These findings were subsequently confirmed by Roblin et al. (1945) who reported essentially similar antimethionine activity by ethionine and methoxinine. The growth of *Escherichia coli* and *Staphylococcus aureus* in synthetic media was prevented by ethionine and this action was reversed by L-methionine but not by the D-isomer. Neither p-amino

benzoic acid nor choline alone counteracted ethionine but the two in combination brought about a partial reversal. In combination with sulfonamides, one fourth of the minimum effective concentration of ethionine together with one-fourth the minimum effective concentration of a sulfonamide produced complete bacteriostasis.

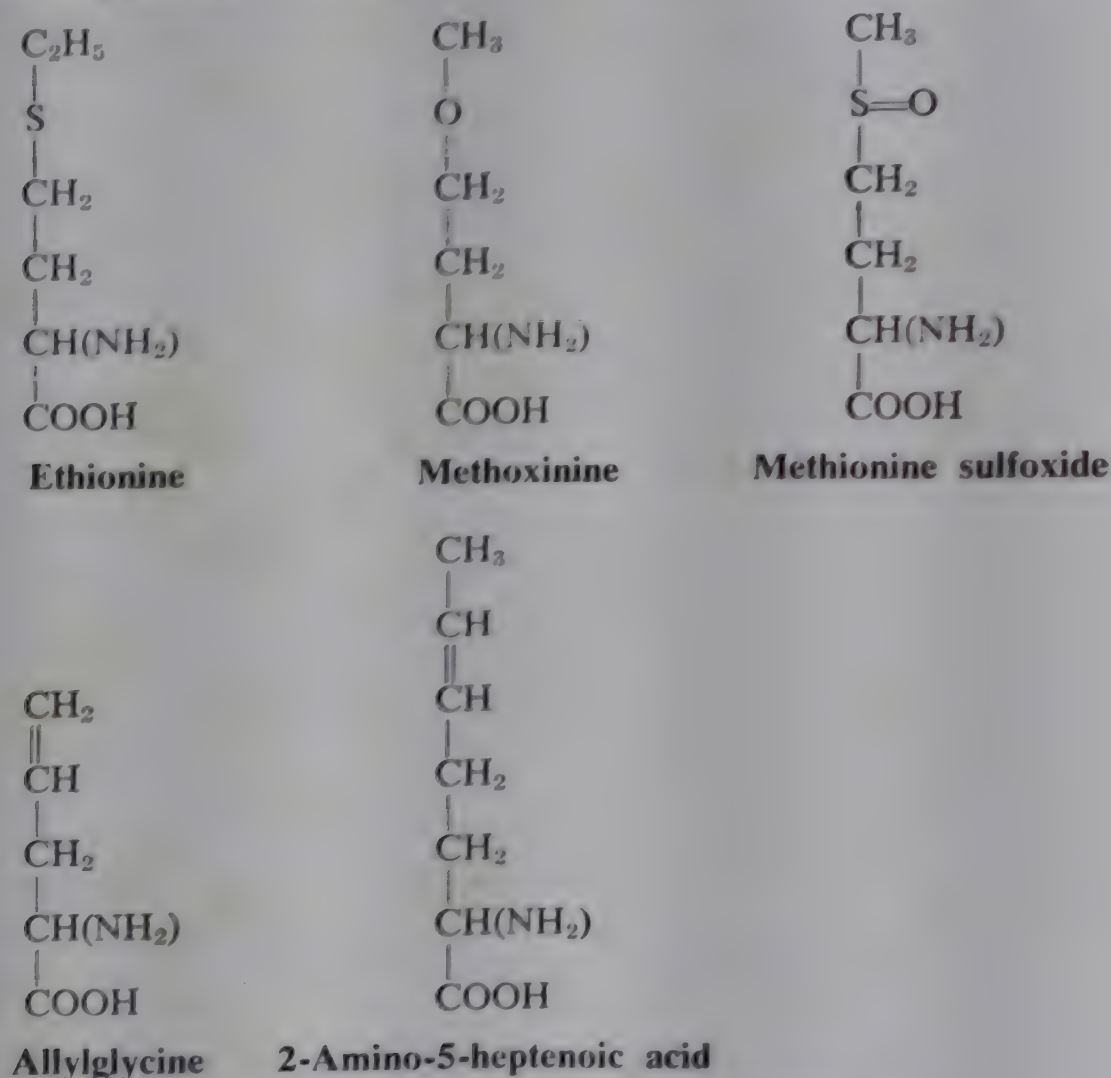


FIG. 2. Metabolite analogues of methionine.

In studies on mammalian metabolism, ethionine was shown to exert a toxic effect as reflected in weight loss in rats on low methionine diets (Dyer, 1938) while it prevented the accumulation of liver fat and the hemorrhagic kidney degeneration in rats on low methionine, low choline diets (Hardwick and Winzler, 1948). The latter authors observed that the action of ethionine in its protective aspects did not seem to stem from direct participation in phospholipid formation. Further confirmation of the protective as well as the deleterious actions of ethionine in the rat was presented by Stekol and Weiss (1949) who found that all optical forms of ethionine acted in the same manner and that this activity was reversed by L-methionine or D-methionine. It will be recalled that both optical antipodes of methionine are effective in the rat. In general, the association of optical activity with

displacement and with the reversal thereof depends entirely upon the availability of the isomers for the nutrition of the microorganism or animal which is the test system. If both optical forms are nutritionally equivalent, then both optical forms of the metabolite analogue will be equivalent as displacers. Stekol and Weiss also reported the failure of cystine, homocystine, and cystathionine in the reversal of the growth inhibition produced by ethionine. Choline was effective.

A logical explanation of the growth inhibitor effect of ethionine was presented by Simpson et al. (1950) when they offered as the cause the competitive inhibition by the antimetabolite of the incorporation of methionine into the proteins of the intact rat. Glycine as well as methionine was prevented from undergoing enzymatic processes associated with protein synthesis. The radioactive sulfur contained in the methionine used by these investigators made it possible to determine rates of incorporation into various tissue proteins. The effect of ethionine was greater when the testing system was liver than when it was kidney. Shen and Lewis (1946) had observed the metabolic inertness of ethionine but recorded the formation of keto acid. Simpson et al. propose as an explanation of the liver-kidney difference that the kidney eliminates ethionine more rapidly either by oxidative deamination or by excretion or both. These investigators also observed inhibition by ethionine of the conversion of methionine sulfur to cystine sulfur. This finding probably bears no relation to the growth inhibiting effect of ethionine as Stekol and Weiss reported the failure of any of the intermediates in the conversion of methionine to cystine to counteract the antimetabolite. If in a given experiment, limitation of both cystine and methionine were critical for protein formation and growth, then it is likely that the growth inhibitory effect of ethionine would be partially reversed by cystine.

Simpson et al. (1950) speculate upon the potentiality that there is complete *de novo* synthesis of protein which would cause any given effective metabolite analogue of an amino acid to inhibit the uptake of all amino acids indiscriminately. If this follows, potential chemotherapeutic and pharmacological agents should be designed in accordance with their possible capacity to interfere with specific precursors or products of the selective metabolism of amino acids rather than with their incorporation into protein or any phase of their group metabolism which would be such a general phenomenon and render improbable specific action.

By modification in the experimental technic of Hardwick and Winzler (1948), for the demonstration of the lipotropic activity of ethionine, Farber et al. (1950) were able to demonstrate induction of fatty livers by this antimethionine. Methionine alone counteracted the observed effect; choline and other lipotropic agents were essentially inactive. The failure of choline to reverse the induction of fatty livers by ethionine is interpreted as a

demonstration of a role played by methionine in lipid metabolism which is distinct from its capacity to serve as a donor of labile methyl for the synthesis of choline. The administration of carbohydrate, in large doses, was observed to prevent or cure the fatty livers. Ethionine fatty livers may, therefore, be starvation fatty livers.

Farber et al. (1950) point out the differences in molar ratios of metabolite/antimetabolite preventing the effect of ethionine in the three systems studied: fatty livers, $\frac{1}{4}$; inhibition of incorporation of amino acids into liver protein, $\frac{1}{1}$; and conversion of methionine-S to cystine-S, $\frac{2}{1}$. Interpreted in the reverse manner, it would indicate that increasing ethionine in the animal body would first inhibit the conversion of methionine to cystine, then prevent protein formation, and finally as the concentration of ethionine reached a point at which it exceeded that of methionine by a factor of four, fatty livers would develop. This is an extremely important observation viewed in its broader implications since it suggests that any given metabolite antagonist may precipitate one possibly desirable effect of blockage of metabolism at a level which would not cause a second and possibly undesirable action.

Direct determinations of the inhibitory effect of ethionine on transmethylation reactions have recently been reported by Simmonds et al. (1950). There was under the experimental conditions a 20 per cent reduction in the amount of transmethylation from methionine to choline; no reduction in the amount of creatine synthesized from dietary methionine was noted. These experiments were carried out using deuterio-DL-methionine and determining the deuteriomethyl content of tissue choline and creatine.

Ethionine therefore interferes with (1) transmethylation, (2) incorporation of amino acids in proteins, (3) conversion of methionine to cystine, and (4) the lipotropic activity of methionine. Choline counteracts the growth inhibiting effect of ethionine (Stekol and Weiss, 1949) but not its induction of fatty livers (Farber et al., 1950).

It seems probable that the effect of ethionine in a given testing system will vary depending upon the limiting factor. For example, the limiting factor might be cystine, methionine, choline, or other related metabolite. Counteraction of the antimetabolite could be effected with any one of these agents depending upon which one is present in critical amounts. On the other hand, if all are present in critical amounts, then it is only logical to expect that counteraction of the inhibitor will be partial with any one of the metabolites and complete with all. With any given metabolite analogue even if 100 per cent specificity were obtained for a given factor—specificity restricting its displacement activity to a single molecule—the overall specificity would be only relative because counteraction would result by supplying the products of the inhibited metabolic systems, etc. Theoretically, this

would not hold if the ultimate effect of the metabolite were in itself essential to life, not channeled through diverse enzymatic systems.

Methoxinine, the oxygen analogue of methionine, was prepared and tested by Roblin et al. (1945). The results obtained with this molecule were similar to those observed by these investigators with ethionine, and as this material is detailed in the discussion of ethionine it will not be repeated here. Suffice it to say that it was an effective metabolite analogue in bacterial systems and that it exerts a synergistic effect when used in combinations with sulfonamides.

It would seem probable that no differences would be found between the actions of ethionine and methoxinine because in both instances the methionine mercapto radical is modified. It is, however, conceivable that deethylation of ethionine and reamination might occur with resultant methionine formation; this is not possible in the case of methoxinine. Shaffer and Critchfield (1948) established both the supplemental and the direct lipotropic action of this oxygen analogue. They assumed that this represented proof that methoxinine did not interfere with transmethylation reactions, which in light of the work reported with ethionine does not seem a valid conclusion. Ethionine is under certain conditions a lipotropic factor (Hardwick and Wuzler, 1948) and yet inhibits transmethylation reactions (Simmonds et al., 1950).

These investigators (Shaffer and Critchfield) also suggest that the failure of equimolar quantities of methionine to counteract the toxicity of ethionine indicates that the antimetabolite might act in a manner other than the competitive antagonism of methionine. Here, again, this assumption is invalidated by the observation that two moles of methionine are needed to counteract one mole of ethionine where the test system is conversion of methionine to cystine (Farber et al., 1950).

The oxidation products of methionine bear an unusual relationship to the parent molecule. Methionine sulfoxide, which might reasonably have been a displacer for methionine, is actually specifically related in this respect to glutamic acid (Waelisch et al., 1946). In fact, the sulfoxide has the same action as methionine in preventing the development of anemia in mice given toxic chemicals (Benard et al., 1947, 1948). The corresponding sulfone is reported to interfere with the metabolism of methionine as reflected in the accentuation by this compound of anemia (Benard et al., 1948, 1948a).

Announcement of the inhibition of the toxicity of agenzized zein by methionine (Heathcote, 1949) led to the assumption that the methionine portion of the protein molecule was essential for the production of the toxic substance and that the toxic agent might well be a metabolite antagonist of the amino acid. Heathcote (1949) found that the toxicity of methionine sulfoxide and methionine sulfone toward *Leucomystoe mesenteroides*

digible compared to that seen with the crystalline principle from the
 rogen trichloride treated protein. The empirical formula for the toxic
 was determined by two groups (Reiner et al., 1950, Bentley et al.,
 1950) as being $C_2H_5NS_2O_2S$. Bentley et al. (1950) state that the molecule
 the toxic principle is derived from methionine sulfoxide by the addition
 $=NH$ or from methionine sulfone by the replacement of O by $=NH$.
 ey point out that the molecule exhibits diastereoisomerism and that the
 isomeric forms may differ in biological activity.

Table I

METABOLITE ANALOGUES OF METHIONINE

Compound	Ac- tivity	Test System	Comment	Reference
Methionine	+	<i>Escherichia coli</i>	Growth	Harris and Kohn, 1941
"	+	"	"	Roblin et al., 1945
"	+	Rats	Toxicity	Dyer, 1938
"	+	"	"	Stekol & Wenz, 1949
"	+	Tissues	Protein synthesis	Simpson et al., 1950
"	+	Rats	Fatty livers	Farber et al., 1950
"	+	Rats	Methionine— Cystine	Simpson et al., 1950
"	+	Rats	Transmethylation	Sammonds et al., 1950
Methoxymine	+	<i>Escherichia coli</i>	Growth	Harris and Kohn, 1941
"	+	Rats	Toxicity	Snaffer & Critchfield, 1942
Methionine sulfone	+	Rats	Anemia	Benard et al., 1948, 1948a
β-glycine	+	<i>Saccharomyces cerevisiae</i>	Growth	Dollmer et al., 1942
"	+	<i>Escherichia coli</i>	Oxygen consumption	Gordonman et al., 1949
α-amino-5- heptenoic acid	+	<i>Escherichia coli</i>	Growth	Goering et al., 1948
β-leucine	+	<i>Escherichia coli</i>	Growth	Harris and Kohn, 1941
"	+	"	"	Lampen & Jones, 1947
"	+	<i>Proteus morganii</i>	Growth	Porter & Meyers, 1945
Monamides	+	Bacteria	Non-specific, secondary	Harris and Kohn, 1941
Chloro-4- aminobenzoic acid	+	Bacteria	Non-specific, secondary	Stave and Roberts, 1946

Pharmacological potentialities for methionine displacers are reflected in the findings of Reiner et al. (1950) showing the suppression of toxic factor induced convulsions or delay in their onset by methionine. This action was found in both rabbits and mice. The convulsions produced by Metrazol and nikethamide were not reversed by methionine, an observation which in no way reduces the possibilities for the use of methionine displacing agents as analeptics.

Metabolite Analogues Produced by Molecular Modifications Other than as in Section §1. Also, Miscellaneous Inhibitors

Unsaturated amino acid analogues of methionine have been prepared and found effective as antagonists. Allylglycine, methylallylglycine, and crotylglycine were inhibitory to the growth of *Saccharomyces cerevisiae* (Dittmer et al., 1948). Goodman et al. (1949) subsequently found that allylglycine retarded the rate of oxygen consumption by *Escherichia coli*, an action reversed completely by methionine.

Another unsaturated molecule, 2-amino-5-heptenoic acid, which is the vinylene analogue of methionine, has been reported to inhibit the growth of *Escherichia coli* (Goering et al., 1948), the effect being reversed by methionine. DL-N-Monomethylmethionine fails as an antagonist, being oxidatively deaminated and metabolized as readily as is methionine (Shen and Lewis, 1946).

Selenium has attracted attention due primarily to its poisoning effect on cattle. Protection against the toxicity of selenium salts is afforded by proteins and specifically by methionine (Lewis et al., 1940), as cystine did not protect. The implication contained in such observations is that selenium might cause the formation of a methionine displacing agent. The case is fortified by the specificity of methionine in reversing the toxicity of selenate for yeast. In the studies of Fels and Cheldelin (1948), no other compound possessed this capacity and, within limits, the growth of yeast in the presence of the selenate was directly proportional to the methionine present. The findings of Moxon et al. (1940) which indicate that selenium may take the place of sulfur in the synthesis of mercapturic acids, lend further support to the displacer formation concept of selenium poisoning.

Recently, the selenium analogues of methionine and homocystine have been synthesized (Painter, 1947; Klosterman and Painter, 1947).

Thallium toxicity may also have its basis in methionine metabolism. Gross et al. (1948) demonstrated that the addition of 2 per cent methionine was less effective than 1 per cent cystine in delaying the onset of the manifestation of thallotoxicosis. They interpreted this experimental fact to indicate a blockage by thallium of the conversion of methionine to cystine or of the conversion of the unnatural form of methionine to the natural. The more probable explanation offered was that thallium combined with homocystine

methionine sulfhydryl thus preventing further participation by this radical in metabolic transformations.

The possibility remains that displacing agents may play a role in the functioning of soybean growth inhibitors. This would be indirect and related to retardation of the release of methionine from protein caused by the soybean trypsin inhibitor (Liener et al., 1949).

Finally, in the class of metabolite analogues produced by molecular modification of methionine at a point other than the methylmercapto radical, certain other amino acids must be placed. Nielsen (1941) noted the toxicity of methionine as tested on yeast, an action prevented by β -alanine. In this same vein, Harris and Kohn (1941) reported the prevention of the toxicity of norleucine for *Escherichia coli* by methionine. This action was frequently established to be competitive in nature when *Proteus morganii* was the test organism (Porter and Meyers, 1945) and also in an *Escherichia coli* system (Lampen and Jones, 1947).

Recently, Harding and Shive (1948) have applied the technique of inhibition analysis to the study of the interrelationships of methionine and leucine. In this paper, they list four types of reversing agents other than the metabolite in competitive displacement inhibitions: (a) precursors of the metabolite, (b) product of the enzymatic action retarded by the analogue, (c) substances exerting a sparging action on the product of the reaction, and (d) chemicals causing an increase in the effective enzyme concentrations. Norleucine was the only competitive antagonist for methionine, but threonine and homocystine counteracted the inhibition in a manner suggesting that these substances may be precursors of methionine (type a) reversing agents). Leucine, isoleucine, and valine tended to reverse the action of norleucine in a manner indicative of the functioning of methionine in the biosynthesis of these amino acids (type (c) reversing agents). In still another category, α -ketoglutaric acid, glutamic acid, panthothenic acid, and biamine occurred and seemed to function by increasing the effective enzyme concentration blocked by norleucine (type (d) reversing agents).

Methionine and the Sulfonamides

The ability of methionine to counteract the action of sulfonamides on *Escherichia coli* was first reported by Harris and Kohn (1941) and by Bliss and Long (1941). This action of methionine is not one of competitive inhibition but reflects the capacity of the sulfonamides to prevent methionine synthesis. Study of the antibacterial action of 2-chloro-4-aminobenzoic acid has clarified the mechanism of action of sulfonamides on methionine synthesis. Shive and Roberts (1946) suggest that the sulfonamides interfere with the action of many enzyme systems but that the most sensitive is that responsible for the synthesis of methionine. They found that the bacteriostatic action of 2-chloro-4-aminobenzoic acid was completely nullified by

methionine, which observation they interpret as indicating selective action of the acid on the enzyme system involved in methionine synthesis. These contentions are supported by the work of Strandkov (1947) who emphasized the continued susceptibility to sulfonamides of bacteria resistant to the action of 2-chloro-4-aminobenzoic acid. Sulfonamide resistant bacteria are not susceptible to the action of the acid.

The possibility that p-aminobenzoic acid causes increased formation of methionine by some direct transformation has not been eliminated and the partial counteraction of the action of ethionine by a combination of choline and p-aminobenzoic acid (Roblin et al., 1945) tends to support this contention. The increased synthesis of p-aminobenzoic acid resulting from added methionine does not seem to have been eliminated although it certainly is not the critical metabolic channel affected in the sulfonamide counteraction by methionine.

Two laboratories (Winkler and deHaan, 1948; Strehler, 1950) have presented evidence indicating the methylation of homocysteine as the point of action of p-aminobenzoic acid in the synthesis of methionine. The site of sulfonamide action on this system would be identical. Methionine only partially counteracted a sulfonamide, which lends further support to the multiplicity of action sites of this drug.

Enzymatic Aspects

In addition to the points of enzymatic inhibition mentioned before, it is to be anticipated that methionine analogues will reduce succinic dehydrogenase activity and completely eliminate that of liver xanthine oxidase. This assertion is based upon the observation that such enzymatic defects are produced by a dietary methionine deficiency (Williams et al., 1949).

Recapitulation

Molecular modifications of the methionine molecule involving the methylmercapto radical are effective antimetabolites. Ethionine, typical of these displacers, interferes with transmethylation, incorporation of amino acids into proteins, conversion of methionine to cystine and the lipotropic activity of methionine.

Two points of paramount importance in the study of metabolite analogues of amino acids are illustrated by the antimethionines. The first of these concerns the probability that most metabolite analogues of amino acids will at some concentration interfere with the general metabolism of all amino acids as reflected in protein synthesis. Ethionine prevents the incorporation of methionine into proteins and it also exerts a similar action on glycine. If this concept is correct, the design of amino acid analogues must concern itself for practical application with the second point, which is the differential efficacy of a given antimetabolite against the group reac-

tions of amino acids and the specific attributes of a given amino acid. Fortunately, it is probable that such differentials exist. In the case of the methionine antimetabolites, an increasing concentration of ethionine will first inhibit the conversion of methionine to cystine and then prevent protein formation. This represents an instance in which a given amino acid displacer interferes primarily with a specific reaction (conversion of methionine to cystine) of an amino acid and only with increased concentrations interferes with group reactions of amino acids (protein synthesis). It seems probable that at least all of the essential amino acids will exhibit multiple specific and individual functions not concerned with group phenomena of amino acids.

The synergism manifested between sulfonamides and ethionine or methoxinine indicates the efficacy of the use of a displacing agent primarily designed to inhibit the specific metabolite with a metabolite analogue designed to displace the secondary inhibitors of the primary displacer. p-Aminobenzoic acid is involved in mechanisms resulting in the formation of methionine. A sulfonamide has as its prime target p-aminobenzoic acid; methionine which is the product of an inhibited enzyme reaction functions as a secondary inhibitor of the action of the sulfonamide. The use of ethionine to eliminate the counteraction of the methionine for the sulfonamide greatly enhances the activity of the primary inhibitor, the sulfonamide. It is proposed that maximum chemotherapeutic efficacy will result from the proper combination of displacing agents to inhibit not only the primary but also the secondary metabolites involved in any given enzymatic concatenation. There would seem to be no practical limit to the number of agents giving increased effect. When the possible permutations and combinations of such inhibitors are considered, it will be clearly seen how limited has been such study to date.

It is probable that the primary directly competitive inhibition which is modified by the secondary noncompetitive reversing agent will be augmented by the inclusion of a competitive inhibitor for the secondary reversing agent. p-Aminobenzoic acid is a competitive inhibitor of a sulfonamide; methionine is a secondary noncompetitive inhibitor of a sulfonamide; ethionine which is a competitive inhibitor of methionine augments the chemotherapeutic efficacy of the sulfonamide. This concept has general application.

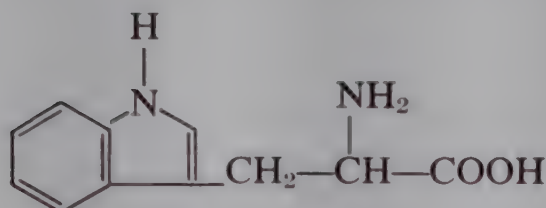
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Chapter 6

TRYPTOPHAN ANALOGUES



Tryptophan
 α -Amino-3-indolepropionic acid

1. Metabolite Analogues Produced by Ring Substitution.
2. Metabolite Analogues Produced by Ring Modification.
3. Metabolite Analogues Produced by Alanine Side Chain Modification; Metabolite Analogues of Indole, Miscellaneous.
4. Metabolite Analogues of Anthranilic Acid.
5. Enzymatic Aspects.
6. Chemotherapy and Pharmacology, Including All Animal Studies.
7. Recapitulation.

Tryptophan, which makes up from 0.5 to 2.5 per cent of animal protein, is one of the essential amino acids, occurring in nature as the L-form; it is not found free. Absence of this amino acid from certain vegetable proteins made it possible to produce deficiency states in animals and the observation followed that a tryptophan free diet caused negative nitrogen balance in the mouse, rat, dog, chicken, and man. The deficiency syndrome is characterized by loss of appetite and weight, reduction in serum albumin and globulin, sterility, loss of hair, hardening of the skin, development of cataracts, and finally death.

Tryptophan undergoes putrefaction in the intestines with resultant formation of several toxic substances—indole, skatole, and 3-(2-aminoethyl) indole—tryptamine. The latter compound has a powerful physiological action and is a violent poison.

Metabolic pathways of tryptophan are multiple and involve delicately balanced interrelationships. The unnatural D-form of the amino acid is utilized by the rat, to a certain extent by the chick, but is not assimilated by man and leads to the excretion of an "aberrant metabolite." Rats, guinea pigs, dogs, and coyotes synthesize kynurenic acid from L-tryptophan but

methyltryptophan inhibited the growth of *Escherichia coli* and he observed the reversal of this action by tryptophan. It was proposed by Anderson that these results explained the observations of Gordon and Jackson (1935) who found this analogue and 2-methyltryptophan toxic to animals on diets containing traces of tryptophan. An interesting finding presented in the Anderson paper is that D,L-5-methyltryptophan has the same activity as tryptophan as a cofactor for the adsorption of viruses onto the host cells. Extending his initial observations, Anderson (1945a) reported that D,L-phenylalanine, D,L-tyrosine, d,l-diiodotyrosine, and 2-methyltryptophane (D,L-Py-2-methyltryptophan) were active as cofactors. From the inactivity of some 47 other compounds tested, he concluded that a tryptophan analogue must have an intact α -amino group (indole-3-propionic acid and amino-N-methyltryptophan being inactive), must have a free carboxyl group (tryptamine and 3-(3-indolyl)-2-propanol were inactive), and must have an l-configuration (d-tryptophan was inactive). His conclusion states "the process of adsorption involves something more than the simple fitting of structural parts of virus and host; a chemical reaction involving the cofactor may well have to follow the chance encounters between virus and host for the virus to be adsorbed and initiate its activity on the host."

The virus work of Anderson is discussed at this point to emphasize the correlation of displacement with chemotherapy in virus disease states.

Fildes and Rydon (1947) report inhibition of growth of *Bacterium*

Table I

METABOLITE ANALOGUES OF TRYPTOPHAN PRODUCED BY
RING SUBSTITUTION

Compound	Ac- tivity	Microörganism	Specific Action	Reference
D,L-5-Methyl- tryptophan	+	<i>Escherichia coli</i>	Growth	Anderson, 1945
	—	<i>Escherichia coli</i>	Growth	Fildes & Rydon, 1947
	—	<i>Escherichia coli</i>	Growth	Ellinger & Kader, 1949
	+	<i>Escherichia coli</i>	Conversion— ornithine to nicotinamide	" "
2-Methyl- tryptophan . .	+	<i>Escherichia coli</i>	Ornithine→ nicotinamide	Ellinger & Kader, 1949
4-Methyl- tryptophan . .	+	<i>Escherichia coli</i>	Ornithine→ nicotinamide	Ellinger & Kader, 1949
7-Methyl- tryptophan . .	+	<i>Escherichia coli</i>	Ornithine→ nicotinamide	Ellinger & Kader, 1949
	+	<i>Lactobacillus arabinosus</i>	Growth	" "
D,L-6-Methyl- tryptophan . .	Not re- ported			Snyder & Pilgrim, 1948
5-Fluoro-DL- tryptophan . .	Not re- ported			Rinderknecht & Niemann, 1950

typhosum by various methyltryptophans with methyl groups in the indole nucleus. Tryptophan is an essential nutrient for this microorganism. In contrast to the observations of Anderson (1945), they report that *Escherichia coli*, which does not need tryptophan for growth, was not inhibited by methyltryptophans. The conclusion was reached that methyltryptophans prevent the use of tryptophan in protein synthesis. The degree of potency of any given molecule depended on the position of the substitution.

The effect of 2-, 4-, 5-, and 7-methyltryptophans and of abrine on growth and nicotinamide formation by pure cultures of *Escherichia coli* and mixed cultures and on the acid production by *Lactobacillus arabinosus* was studied by Ellinger and Kader (1949). In confirmation of the work of Fildes and Rydon, they found that the growth of *Escherichia coli* or of mixed cultures was not markedly affected by any one of the methylated tryptophans. By contrast, the growth of *Lactobacillus arabinosus* was considerably inhibited by the 7-methyl compound. In spite of their failure to inhibit growth, all methylated compounds inhibited nicotinamide synthesis by *Escherichia coli*. Abrine did not inhibit.

6-Methyltryptophan (Snyder and Pilgrim, 1948) and 5-fluoro-DL-tryptophan (Rinderknecht and Niemann, 1950) have been synthesized but as yet no report has been made on their biological activity.

Metabolite Analogues Produced by Ring Modification

The creation of analogues in this category might involve modification in the benzene or in the pyrrole rings. The first attempt to modify the indole nucleus involved replacing the —NH— radical in the pyrrole ring by a vinylene group. Dittmer et al. (1948) prepared β -1- and β -2-naphthylalanines which had been synthesized by Kikkoji (1911) and found that neither compound inhibited growth of *Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Lactobacillus casei*, or *L. arabinosus*.

In 1948, Avakian et al., prepared and tested β -(2-benzothienyl)- α -aminopropionic acid, also designated as β -3-thianaphthenylalanine. The compound was effective as a tryptophan displacer when *Lactobacillus arabinosus* was the test organism. The inhibitor-metabolite ratio for the compound was 250; the corresponding figure for 5-methyltryptophan was 2500. Working independently, Elliott and Harington (1949) prepared the same analogue and found that it was active when *Streptococcus hemolyticus* was the test organism but was not inhibitory to *Staphylococcus aureus*, or *Escherichia coli*.

A third molecular species representing a potential metabolite analogue of tryptophan was prepared by Erlenmeyer and Grubenmann (1947). The molecule was β -(coumaronyl-(3))-alanine which is the oxygen analogue of tryptophan. It may be assumed that the molecule was not active as no report has been made.

Table II

METABOLITE ANALOGUES OF TRYPTOPHAN PRODUCED BY RING MODIFICATION

Compound	Ac- tivity	Microorganism	Specific Action	Reference
β -1-Naphthyl- alanine	—	<i>Escherichia coli</i> <i>Saccharomyces cerevisiae</i> <i>Neurospora</i> <i>Lactobacillus casei</i> <i>Lactobacillus arabinosus</i>	Growth	Dittmer et al., 1948
β -2-Naphthyl- alanine	—	" " " "	Growth	Dittmer et al., 1948
β -3-Thianaph- thenylalanine	+	<i>Lactobacillus arabinosus</i>	Growth	Avakian et al., 1948
	+	<i>Streptococcus hemolyticus</i>	Growth	Elliott & Harington, 1949
	—	<i>Escherichia coli</i>	Growth	" "
β -(Coumaronyl- (3))-alanine	Not re- ported			Erlenmeyer and Grubenmann, 1947

Metabolite Analogues Produced by Alanine Side Chain Modification; Metabolite Analogues of Indole, Miscellaneous

To Fildes (1941) goes the credit for making the first tryptophan metabolite analogues. He found that traces of tryptophan prevented the inhibitory action of indoleacrylic acid on bacteria and postulated from this that the activity was due to the inhibition of the synthesis of tryptophan. There was no quantitative relationship between indoleacrylic acid and indole, which is a known precursor of this essential amino acid. Fildes suggested that some other stage of metabolism of indole to tryptophan, represented by a compound X, was the point of inhibition. He had previously pointed out that indoleacrylic acid actually stimulated the production of indole from tryptophan by the enzyme contained in *Escherichia coli* (Fildes, 1938). These combined observations indicate that in indoleacrylic acid we have a molecule which prevents the formation of a given metabolite and stimulates its destruction. It seems not impossible that the action of a given compound in a system of the type under consideration might be to stimulate destruction of a given essential metabolite to such an extent as would seriously interfere with the growth potential of bacteria.

In 1942, Bloch and Erlenmeyer tested β -(naphthyl-(1))acrylic acid, styrylacetic acid, cinnamic, dihydrocinnamic, benzoic, and fumaric acids in the same systems used by Fildes (1941). Styrylacetic and naphthylacrylic acids were similar to indoleacrylic acid. Cinnamic acid had a weakly in-

hibitory effect while benzoic, fumaric, and dihydrocinnamic acids had no action whatever. The agents were tested against *Escherichia coli* in a simple synthetic medium containing no organic nitrogen other than tryptophan.

The action of indoleacrylic acid is apparently not specific, as Marnay (1949) has demonstrated the capacity of phenylalanine to counteract the analogue. Using a strain of *Escherichia coli*, she found that the growth inhibiting effects of 10^{-4} M. of the antimetabolite were overcome by 5×10^{-4} M. of tryptophan, 1.5×10^{-4} M. of nicotinic acid and 6×10^{-4} M. of L-phenylalanine. Another recent study of the action of indoleacrylic acid is that of Furst and Harper (1949) who found that this compound did inhibit the growth of *Lactobacillus arabinosus* or *Leuconostoc mesenteroides* and that reversal occurred with added tryptophan. Using a lactobacillus which did not require an exogenous source of the nutriline and which presumably synthesizes it, actual stimulation was noted. The authors suggest that indole-

Table III

METABOLITE ANALOGUES PRODUCED BY ALANINE SIDE CHAIN MODIFICATION
INDOLE METABOLITE ANALOGUES, MISCELLANEOUS

Compound	Activity	Microorganism or	Specific Action	Reference
Abrine, N-methyl- tryptophan . .	—	<i>Escherichia coli</i>	Ornithine→ nicotinamide	Ellinger & Kader, 1949
β -(Indole-(3)) acrylic acid . .	+	<i>Escherichia coli</i>	Growth	Fildes, 1941
	+	<i>Escherichia coli</i>	Growth	Bloch and Erlenmeyer, 1942
β -(Naphthyl-(1)) acrylic acid . .	+	<i>Escherichia coli</i>	Growth	"
Styrylacetic acid	+	<i>Escherichia coli</i>	Growth	"
Cinnamic acid . .	+	<i>Escherichia coli</i>	Growth	"
	—			
Benzoic acid . . .	—	<i>Escherichia coli</i>	Growth	"
Fumaric acid . .	—	<i>Escherichia coli</i>	Growth	"
Dihydrocin- namic acid . . .	—	<i>Escherichia coli</i>	Growth	"
β -(2-Quinolyl)- acrylic acid . .	+	<i>Lactobacillus</i>	Growth	Furst & Harper, 1949
β -(4-Quinolyl)- acrylic acid . .	+	"	"	" "
β -(3-Quinolyl)- acrylic acid . .	—	"	"	" "
β -(6-Quinolyl)- acrylic acid . .	—	"	"	" "
DL-Homotryp- tophan	Not re- ported			Snyder & Pilgrim, 1948a
3-Acetyl- pyridine . .	+	Mouse	Growth	Woolley, 1946

acrylic acid supplied a preformed indole nucleus for tryptophan manufacture. Four quinoline acrylic acids were included in this study and only two— β -(2-quinolyl) acrylic and β -(4-quinolyl) acrylic acids—were effective competitive inhibitors of tryptophan.

Fildes and Rydon (1947) studied not only methylated tryptophans but also the corresponding indoles. In general, these indoles obstructed the synthesis of tryptophan from indole, just as the methylated tryptophans inhibited the use of tryptophan in protein synthesis.

An announcement was made of the synthesis of DL-homotryptophan (Snyder and Pilgrim, 1948a) but to date no report has followed concerning its biological activity, which may well mean that it proved ineffective.

3-Acetylpyridine must be placed (Woolley, 1946) in the category of miscellaneous antimetabolites for tryptophan. The amino acid nullified the toxicity of the antimetabolite, completely preventing the development of pellagra-like symptoms. Tryptophan was as active as nicotinic acid in counteracting 3-acetylpyridine—0.1 per cent of the amino acid protected animals against 4 mg. of the analogue.

Metabolite Analogues of Anthranilic Acid

Anthranilic acid is involved in the biosynthesis of tryptophan by microorganisms and therefore is a logical target for the design of displacers. Rydon (1948) studied a series of such agents and reported that 4- and 5-methylanthranilic acids were potent growth inhibitors of *Bacterium typhosum*, which synthesizes anthranilic acid, and does not produce it by degradation from indole or tryptophan. The inhibition produced by these chemicals was reversed equally well by anthranilic acid, indole, and tryptophan, but not by p-aminobenzoic acid. The author concluded that the methylated anthranilic acids act by inhibiting the conversion of anthranilic acid to indole.

Table IV

METABOLITE ANALOGUES OF ANTHRANILIC ACID

Compound	Activity	Microorganism	Specific Action	Reference
4-Methylan- thranilic acid . .	+	<i>Bacterium typhosum</i>	Anthranilic→ indole	Rydon, 1948
5-Methylan- thranilic acid . .	+	<i>Bacterium typhosum</i>	Anthranilic→ indole	Rydon, 1948

Enzymatic Aspects

Two major phases of tryptophan metabolism have been studied from the standpoint of the action of antimetabolites on the enzymes involved. The first of these concerns the biosynthetic aspects of the enzymology of tryptophan metabolism. Here, in brief, the enzymatic conversion of an-

thranilic acid to indole is inhibited by the methylated anthranilic acids, the conversion of indole to tryptophan is prevented by the methylated indoles and by indoleacrylic acid, and the final phase which is that of enzymatic incorporation of tryptophan into protein is blocked by the methylated tryptophans. Details have been given in the preceding discussion and will not be repeated here.

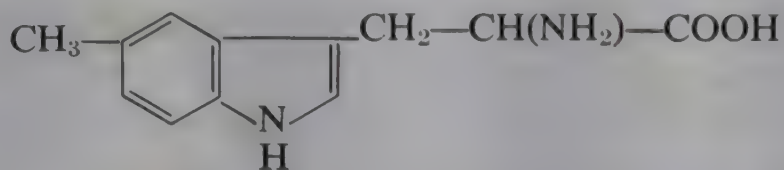
The second phase concerns the enzymatic breakdown of the amino acid by the enzyme, tryptophanase. It will be recalled that this enzyme catalyzes the conversion of tryptophan to indole, pyruvic acid, and ammonia. The first study of inhibition of this system was that of Tatum and Bonner (1944) who observed the effectiveness of serine in preventing the formation of indole. The inhibition was directly proportional to the serine concentration and a mass action effect was suggested. Dawes et al. (1947) studied the problem of inhibitors of tryptophanase and found both serine and alanine effective. The action was not a simple relation between the tryptophan and serine concentrations, and only occurred when the enzyme was saturated with tryptophan. The suggestion of Tatum and Bonner (1944) concerning mass action effect applied only when tryptophanase was fully saturated with tryptophan.

Chemotherapy and Pharmacology, Including All Animal Studies

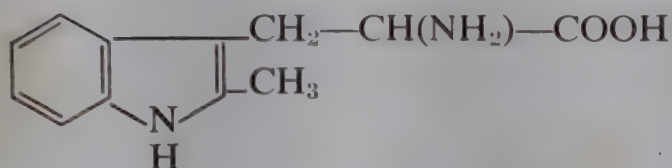
The proposal that conversion of tryptophan into nicotinic acid in the rat is inhibited by indole-3-acetic acid (Kodicek et al., 1946) has been proved incorrect by numerous investigators (Krehl et al., 1947; Rosen and Perlzweig, 1947; and Sarett and Goldsmith, 1947).

Analogues of tryptophan which function by inhibiting its formation are of particular importance in the light of the observations of Sevag and Green (1944a) who noted that pantothenic acid not only mediates the metabolism of glucose leading to or involved in the formation of tryptophan essential for the growth of the exacting strains of *Staphylococcus aureus*, but also functions in the system which in the presence of glucose oxidizes tryptophan to one or more arylamines. If this is accurate then logically the best chemotherapeutic agent against *Staphylococcus aureus* would be one designed to displace tryptophan and prevent its synthesis. Even a combination of a sulfonamide with an agent such as 5-methyltryptophan should prove a superior therapeutic composition.

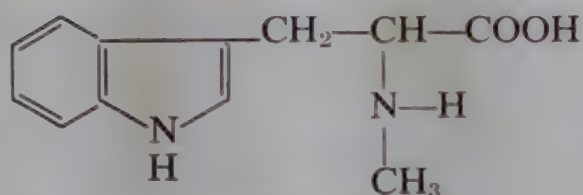
The observations of Anderson (1945a) offer important approaches to the applications of tryptophan analogues in virus chemotherapy. Why does a tryptophan displacing agent effective in a bacterial system for the inhibition of growth act by substituting for the nutritive as the chemical link of virus to host? It would seem that this linkage point offers the direct mode of attack on virus chemotherapy: simply prevent the adsorption. The only reported attempt to utilize this approach to date is that of Delbrück (1948)



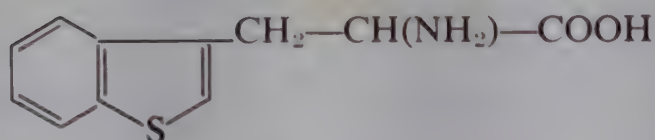
5-Methyltryptophan
(Bz-3-methyltryptophan)



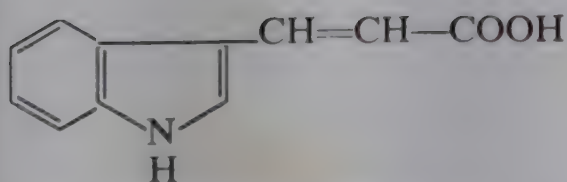
2-Methyltryptophan
(DL-Py-2-methyltryptophan)



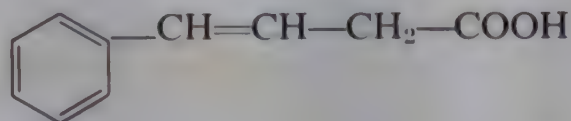
Abrine
L-α-methylamino-β-(3-indolyl)-propionic acid



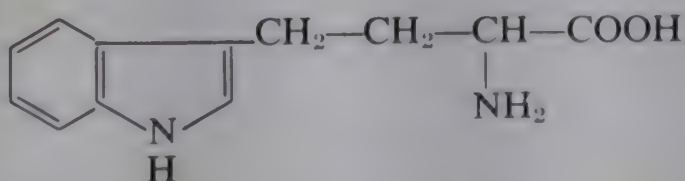
β-3-Thianaphthenylalanine
β-(2-benzothieryl)-α-amino-propionic acid



β-(indole-(3))-acrylic acid



Styrylacetic acid



DL-Homotryptophan

FIG. 2. Metabolite analogues of tryptophan.

who found that indole inhibits the adsorption cofactor activity of tryptophan and phenylalanine in the systems of Anderson in a manner which suggests competitive inhibition. He points out that this represents an instance in which bacteria produce indole from tryptophan reversing the mechanism in self-protection by converting an activator of an invasive pathogen into an inhibitor. Phage resistant bacteria show a loss of ability to synthesize tryptophan.

Recapitulation

The metabolite analogues of the B-complex series have been shown to act in general at three major points of the metabolism of the vitamin: namely, at the synthesis of the factor, at the incorporation of the nutrilit into a cofactor, and finally at the functioning of the enzyme. With the amino acids, there is the same general picture. The antimetabolite prevents the biosynthesis of the amino acid, or it interferes with the incorporation of the amino acid into a protein, or finally it may interfere with the action or activity of a protein. This latter action has not yet been clearly demonstrated but that such proof will be forthcoming may be anticipated.

Specifically, tryptophan metabolite inhibitors are known for every stage in its biosynthesis and for its incorporation into protein. Methylated anthranilic acids inhibit the conversion of anthranilic acid to indole; methylated indoles prevent the formation of tryptophan from indole; and the methylated tryptophans block the incorporation of the amino acid into the complex protein molecule.

The same metabolite analogue which blocks tryptophan synthesis by *Escherichia coli* stimulates the destruction of this amino acid by tryptophanase. Indoleacrylic acid is therefore an example of an antimetabolite which prevents the formation of a given factor and yet facilitates its destruction. This same bacterium, *Escherichia coli*, is able as a defensive mechanism to convert tryptophan, which is the chemical cofactor for virus adsorption, into indole, a molecule which displaces or inhibits this adsorption. These points should lead to the use of tryptophan displacers with purine antimetabolites in the chemotherapy of the virus diseases.

As mentioned in the introduction to this section, the tryptophan molecule offers potentialities as a model for the design of displacers through modifications in the ring systems, through substitution or ring replacement or by change in the alanine side chain. All of these potentialities have been realized—5-methyltryptophan characterizing those with added substituent, β -3-thianaphthenylalanine those with ring change, and indoleacrylic acid those with alanine side chain alteration.

3-Acetylpyridine is an antimetabolite for tryptophan just as nicotinic acid is a natural antagonist for indoleacrylic acid. Tryptophan is a known precursor of nicotinic acid and if this were its sole limiting function in a

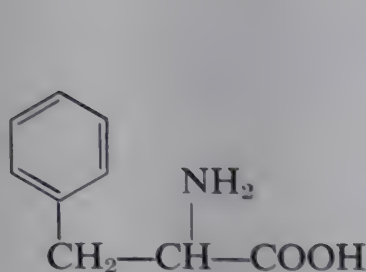
given system, the counteraction of acetylpyridine by the amino acid would be anticipated. But how are we to account for the nullification of the action of indoleacrylic acid by nicotinic acid unless the mechanism is reversible and nicotinic acid functions as a precursor for tryptophan under conditions of metabolic stress?

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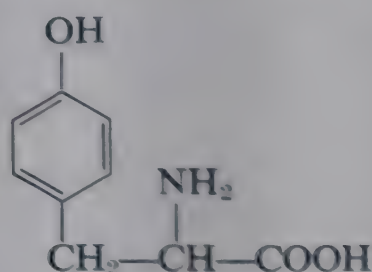
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Chapter 7

PHENYLALANINE-TYROSINE ANALOGUES



Phenylalanine



Tyrosine

1. Metabolite Analogues Produced by Ring Modification.
2. Metabolite Analogues Produced by Ring Substitution.
3. Metabolite Analogues Produced by Alanine Side Chain Modification.
4. Miscellaneous Displacers.
5. Enzymatic Aspects.
6. Chemotherapy and Pharmacology.
7. Recapitulation.

Phenylalanine is an essential amino acid and its oxidation in the animal organism forms tyrosine, a nonessential factor. Isomers of phenylalanine and tyrosine are interchangeable in the dietary of the rat. In man, however, only the natural forms are utilized. Phenylalanine and therefore tyrosine can be replaced in the diet of an animal by acids which have a keto or a hydroxyl instead of an amino group, since amination occurs. Tyrosine lowers the minimal requirements of phenylalanine but cannot replace it. The pharmacological implications of the demonstration that epinephrine is formed from phenylalanine in the animal organism are far reaching and of paramount importance in consideration of the metabolite analogues of this amino acid. The general metabolic pattern for phenylalanine is presented in Fig. 1.

Much information concerning phenylalanine and tyrosine metabolism came from study of inborn errors of the metabolism of these amino acids. Albinism is one of these, characterized by abnormal deficiency in the formation of melanin. The defect is apparently in the absence of an enzyme capable of converting dihydroxyphenylalanine to melanin. Another such metabolic sport is alcaptonuria in which homogentisic acid is excreted. This condition was known in ancient times, the urine of such patients being

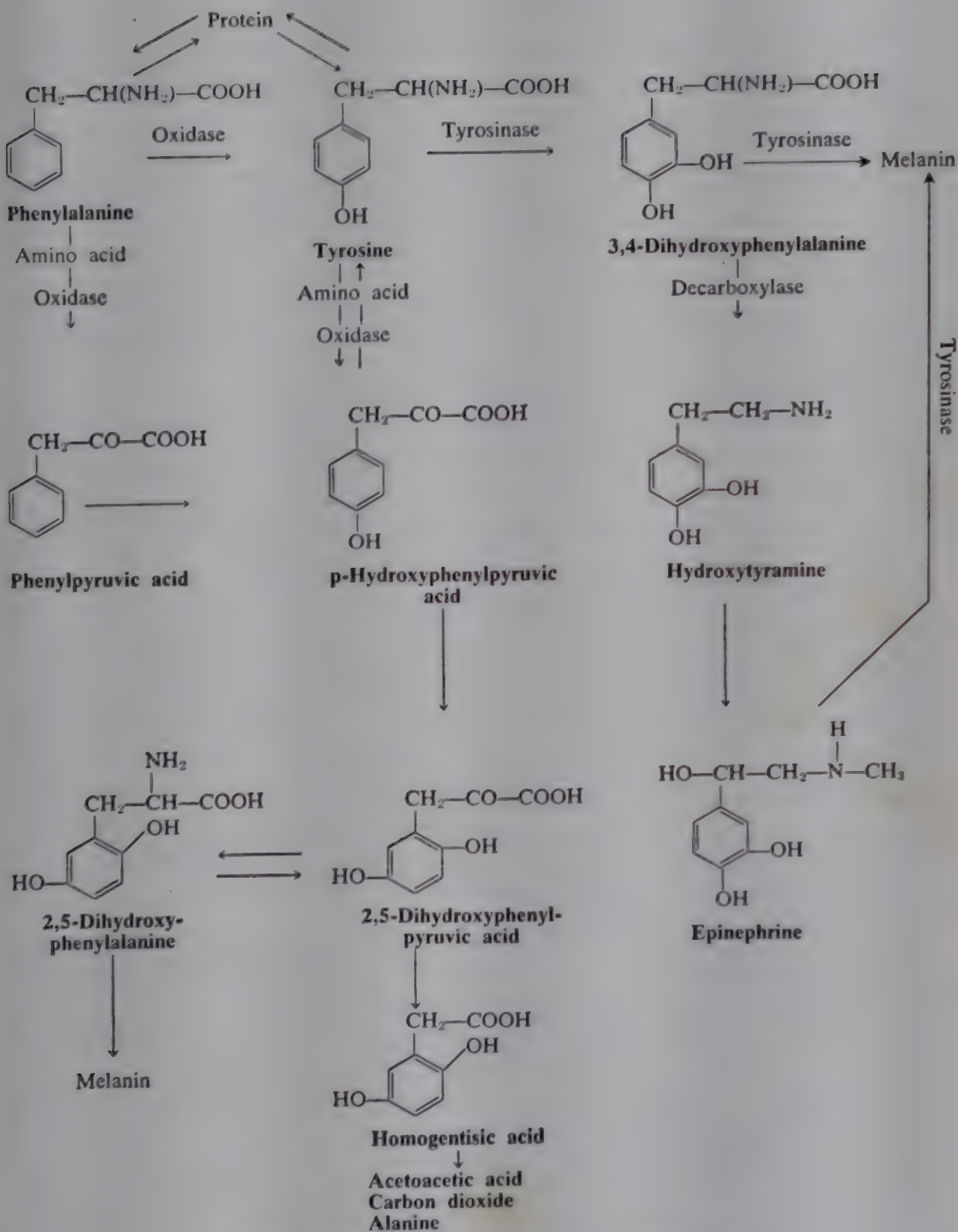


FIG. 1. Phenylalanine-tyrosine metabolism.

described as "black." The alcaptonuric utilizes phenylalanine and tyrosine normally for the production of tissue proteins. Oligophrenia phenylpyruvica is a recent addition to the list. This condition is seen in certain mentally defective patients and is characterized by the excretion of phenylpyruvic acid. The metabolic abnormality is localized at the point of oxidation of phenylalanine to the p-hydroxy compound. Tyrosinosis, another, is characterized by blockage at the next point of tyrosine metabolism, namely p-hydroxyphenylpyruvic acid. Premature infants excrete p-hydroxyphenylpyruvic acid and p-hydroxyphenyllactic acid in the urine, an abnormal state associated with and corrected by ascorbic acid.

These metabolically mutated biochemical states are mentioned here because there is a possibility that some of them may be due to specific naturally occurring metabolite analogues.

The metabolism of these aromatic amino acids is controlled in some manner by ascorbic acid, as manifested by the excretion of homogentisic acid and related molecules in vitamin C deficient guinea pigs and humans. The oxidation of the phenyl nucleus does not occur in the absence of the vitamin. Liver preparations and folic acid also reduce the excretion of aromatic keto acids by vitamin C deficient guinea pigs.

Tyrosine by putrefactive decomposition loses carbon dioxide to form the base tyramine, an active principle of ergot. Tyramine is a powerful physiological agent, raising blood pressure. Although in general, decarboxylation occurs as a result of the action of bacteria in the intestine, there is evidence showing that these amino acids can be degraded in a like manner in vivo. It will be recalled that the phosphorylated derivative of pyridoxal is the cofactor for the decarboxylation of tyrosine. Tyrosine is vital to the metabolic processes which result in the formation of the hormones of the posterior pituitary and thyroxine. The intermediate in the latter conversion is iodogorgoic acid (diiodotyrosine).

Kidney tissue possesses the enzymatic power to convert dihydroxyphenylalanine to hydroxytyramine. The enzyme (dopa decarboxylase) also occurs in liver, small intestine, and in fetal organs. It is active with either the 3,4-acid or the 2,5-dihydroxyphenylalanine as substrate, but not with tyrosine. For the action of dopa decarboxylase, a hydroxyl group in the para- position is not adequate; there must be a hydroxyl, either meta- or ortho-. Two hydroxyls are not needed. Bacteria, specifically, *Streptococcus faecalis*, contain a decarboxylase for tyrosine which acts on dopa and phenylalanine. Both tyrosine and dopa administered in adequate dosage to rats cause hypertension. The mechanism of this action might be through decarboxylation to pressor compounds or through blockage of the action of tyrosinase on epinephrine. Tyrosine poisoning in the rat is characterized by degenerative and necrotizing changes which are most striking in the pancreas.

The phenolic hydroxyl of tyrosine is fundamental to the action of pep-

sin, pepsinogen, insulin, and the chorionic gonadotropic and lactogenic hormones and it is probable that this group contributes to the essential nature of many other protein or peptide catalysts.

The first step in the breakdown of amino acids in the animal organism is deamination, which process is catalyzed by specific amino acid oxidases, most of which are flavoproteins. Oxidases are specific with reference to optical antipodes. L-Amino acid oxidase deaminates phenylalanine and tyrosine. Not only free amino acids but also peptides are deaminated.

Tyrosine is oxidized by the enzyme tyrosinase (a copper-containing protein) to a red compound, which is ultimately transformed into the dark brown pigment known as melanin. At one time it was thought that a "dopa oxidase" distinct from tyrosinase performed the function of the oxidation of dihydroxyphenylalanine to melanin but it is now held that there is but one enzyme involved, namely tyrosinase. Metabolic analogues capable of inhibiting tyrosinase might prevent the inactivation of esterone, estradiol, epinephrine, and similar substances which are normally subject to the action of the enzyme.

DL-Phenylalanine is an activator of penicillinase. In the same vein, dopa activates yeast dipeptidase.

These aromatic amino acids are essential for the optimum growth of several bacteria including *Clostridium sporogenes*, *Corynebacterium diphtheriae*, lactic acid bacteria, *Streptococcus zymogenes*, etc. The bacterial breakdown products of tyrosine include: p-hydroxyphenyllactic acid, p-hydroxyphenylpropionic acid, p-hydroxyphenylacrylic acid, p-hydroxyphenylacetic acid, p-cresol, tyramine, and phenol. *Saccharomyces cerevisiae* and *Escherichia coli* are typical of those microorganisms which do not require phenylalanine in the medium for growth.

N-Methyltyrosine, also called surinamine, occurs naturally and has been used as an astringent. Administered orally, it causes violent purging.

It has been suggested that tyrosine linkage in the protein molecules may be by side chains as well as through the customary peptide union. This assumption was based upon the weaker color reactions given by tyrosine when in an intact protein as contrasted to the total hydrolysate of that protein, but recent evidence indicates that the reacting atomic groups are sterically protected in the protein and so do not react as strongly. Phenylalanine as a unit contributes nonpolar benzyl groups to the protein and tends as a result to decrease the solubility of the protein in water; tyrosine with its hydroxyl radical is polar in character.

As a structural model for the design of metabolite analogues, phenylalanine and tyrosine offer potentialities through the standard pattern of ring change, ring substitution, or alteration in the alanine side chain. As will be seen from the following discussion, examples of each of these possible structural analogue varieties has been synthesized and found effective.

Metabolite Analogues Produced by Ring Modification

The first member of this series to be produced was β -2-thienyl-DL-alanine which was reported by duVigneaud et al. (1945) as inhibiting the growth of *Saccharomyces cerevisiae*, an action nullified by phenylalanine. Tyrosine did not counteract the antimetabolite. Extension of these studies revealed the activity of this molecule against *Escherichia coli*, *Streptococcus faecalis*, and *Lactobacillus arabinosus* (Dittmer et al., 1946). The specificity exhibited by the displacer for the yeast was greater than for a bacterial test system. With the yeast, none of the other amino acids tested was as effective as DL-phenylalanine; however, at concentrations 10 to 30 times as high as that required for phenylalanine counteraction, DL-leucine, DL-isoleucine, DL-tryptophan, DL-methionine, and DL-valine were active. By contrast using *Escherichia coli*, DL-phenylalanine and DL-tryptophan were equally effective and L-tyrosine was also active.

Resolution of β -2-thienyl-DL-alanine by Ferger and duVigneaud (1948) led to the observation that only the L-form possessed inhibitor activity, the D-isomer being inactive. These investigators used a chemical method of resolution. An enzymatic procedure for the resolution of this antimetabolite has been reported by Crowe and Nord (1950).

Beerstecher and Shive (1946) independently applied the same analogue to "inhibition analysis" studies with *Escherichia coli*. The results obtained were identical with those of Dittmer et al. (1946) and were interpreted to indicate that tryptophan was a precursor of phenylalanine.

An improved synthesis of β -2-thienylalanine (Dittmer et al., 1946a) made possible more extensive studies of this molecule. Extension of their studies of β -2-thienylalanine and its effect on the metabolism of *Escherichia coli*, led Beerstecher and Shive (1947) to conclude that this bacterium converts phenylalanine to tyrosine in an essentially irreversible direct oxidation, and that the antimetabolite prevents this oxidation by competing with the natural nutrilit for an enzyme surface. This assumption was based upon the greater effectiveness of tyrosine as contrasted to phenylalanine in counteracting the inhibitor. Logically, the product of an enzyme action should be more effective than a precursor of the normal enzyme substrate in nullifying the action of a metabolite analogue.

Goodman et al. (1949) studied the effect of thienylalanine on bacterial respiration, using the Warburg manometric technic. They found that the antimetabolite markedly inhibited the rate of oxygen consumption by *Escherichia coli* and that this was directly correlated with inhibition of growth. The displacer molecule had no effect on an *Escherichia coli* lactic dehydrogenase system. A 40 per cent inhibition of the activity of dopa decarboxylase is caused by thienylalanine at a level of 2.5 mg./ml. (Martin et al., 1950).

Table 1

METABOLITE ANALOGUES OF TYROSINE-PHENYLALANINE PRODUCED BY RING MODIFICATION

Compound	Action	Test System	Comment	Reference
β -2-Thienyl-DL-alanine . . .	+	<i>Saccharomyces cerevisiae</i>	Growth	duVigneaud et al., 1945
	+	Several bacterial species	"	Dittmer et al., 1946
β -3-Thienyl-DL-alanine . . .	+	<i>Saccharomyces cerevisiae</i> & <i>Escherichia coli</i> .	Growth	Dittmer, 1948, 1949
	+	Tubercle bacillus	"	Pope, 1949
	+	Rat	"	Garst et al., 1949
β -2-Furyl-DL-alanine . . .	+	<i>Saccharomyces cerevisiae</i> & <i>Escherichia coli</i>	Growth	Herz et al., 1947 Clark and Dittmer, 1948
β -2-Pyrryl-DL-alanine . . .	+	<i>Saccharomyces cerevisiae</i> & <i>Escherichia coli</i>	Growth	Herz et al., 1948
β -2-Pyridyl-alanine	+	Phage infected bacteria	Liberation of phage. Not proved anti-phenylalanine	Anderson, 1946
β -3-Pyridyl-alanine	+	Phage infected bacteria	Liberation of phage. Not proved anti-phenylalanine	Anderson, 1946
β -4-Pyridyl-alanine	+	<i>Streptococcus hemolyticus</i>	Growth	Elliott et al., 1948
	+	<i>Escherichia coli</i>	"	"
β -6-Methoxy-quinolyl-4-alanine	+	<i>Streptococcus hemolyticus</i>	Growth	Elliott et al., 1948
	+	<i>Escherichia coli</i>	"	"
β -Quinolyl-4-alanine	—	<i>Streptococcus hemolyticus</i>	Growth	Elliott et al., 1948

The tubercle bacillus which is normally resistant to growth inhibitors was susceptible to the effect of β -2-thienylalanine (Drea, 1948; Pope, 1949), as was the vaccinia virus in chick embryonic tissue (Thompson and Wilkin, 1948). The latter observation can be interpreted to indicate the importance of phenylalanine in the proliferation of the virus. The potentiality remains that the prime point of action might be the host cell rather than the virus particle.

The first and thus far only attempt to ascertain the effect of β -2-thienyl-DL-alanine in mammalian metabolism is that of Ferger and duVigneaud (1949). Two per cent of the antimetabolite added to the diet of the rat

caused an immediate and rapid loss in weight. Any of the optical forms was effective. The authors point out that D-phenylalanine will support the growth of the rat but not of *Lactobacillus delbrueckii*, and with this micro-organism thienyl-D-alanine is inactive. Tyrosine was not effective in counteracting the growth inhibition produced by β -2-thienylalanine. It will be recalled that tyrosine did counteract this antimetabolite when *Escherichia coli* was the test organism but not when *Saccharomyces cerevisiae* was used.

Work with β -2-thienylalanine stimulated interest in the β -3-compound which was synthesized by Campaigne et al. (1948) and by Dittmer (1948, 1949). The latter investigator found the β -3-isomer to be twice as active with yeast as the test system and 30 per cent more active as compared to the β -2-compound if *Escherichia coli* was used. Dittmer proposes the 3-position of the thiophene ring as the point of side chain attachment for obtaining greatest potency in any metabolite analogue designed by replacement of a phenyl ring. β -3-Thienylalanine was the most powerful metabolite analogue tested by Pope (1949) against the tubercle bacillus. Specificity was not great, as not only phenylalanine and tyrosine but also leucine, isoleucine, norleucine, and tryptophan counteracted the toxicity of the antimetabolite.

In mammalian metabolism, the β -3-isomer is also reported to be more powerful than the β -2-compound (Garst et al., 1949). Beyond the quantitative aspects, the results are essentially the same as those obtained with the 2-substituted molecule (Ferber and duVigneaud, 1949). Employing the protein depleted rat, Dittmer et al. (1950) noted inhibition of weight restoration by β -3-thienylalanine, which was reversed by additional amounts of phenylalanine.

Recently, Dunn and Dittmer (1950) have synthesized peptides containing amino acid antagonists such as the two thienylalanines. Glycyl- β -2-thienylalanine, glycyl- β -3-thienylalanine, and β -2-thienylalanylglycine inhibited reversibly the growth of *Escherichia coli*. Quantitatively, these peptide antagonists were less powerful than the corresponding antimetabolites. The β -2-thienylalanine inhibition was reversed by phenylalanylglycine, glycyl-phenylalanine, or glycyl-p-methylphenylalanine. Using yeast as the test organism, these investigators found that the peptides were much less effective than the corresponding free amino acids either as inhibitors or as reversers of inhibition. It would seem open to question whether or not hydrolysis played a role in these observations.

Among other metabolite analogues of phenylalanine-tyrosine designed by ring modification, β -2-furylalanine (Herz et al., 1947) has received considerable study. Clark and Dittmer (1948) found this molecule to be about one-third as good as β -2-thienylalanine as an antagonist. The ability of amino acids to counteract β -2-furylalanine parallels roughly that seen with the thienyl compounds. The furyl compound markedly inhibited the

rate of oxygen consumption of *Escherichia coli* (Goodman et al., 1949). Herz et al. (1948) found β -2-pyrrolylalanine, which they prepared in crude form, to be an antiphenylalanine.

The observation of Anderson (1946) that 2- and 3-pyridylalanine blocked the liberation of active phage particles from infected bacteria seems of paramount importance. The drugs effectively prevented the spread of the phage infection in the bacterial culture confining the infection to cells first attacked. The application of these findings to virus chemotherapy is apparent.

Finally, Elliott et al. (1948) prepared β -4-pyridylalanine, β -4-quinolylalanine, and β -6-methoxyquinolyl-4-alanine; the second molecule listed was the only inactive one. The other two were active against hemolytic streptococci.

Metabolite Analogues Produced by Ring Substitution

Studies with the fluorinated derivatives of phenylalanine and tyrosine had clearly demonstrated their toxicity (Boyer et al., 1941) before the displacement concept was applied. 3-Fluorotyrosine, 3-fluorophenylalanine, 3-fluoro-5-iodotyrosine, and 3,5-difluorotyrosine produced convulsions and hyperirritability followed by extreme lethargy. When fed in the diet at levels as low as 0.0005 per cent, 3-fluorotyrosine inhibited the growth of young rats. While it was not established, it is probable that the toxicological manifestations stemmed from inhibition of some phase of phenylalanine-tyrosine metabolism. Niemann and Rapport (1946) resolved the DL-mixture of 3-fluorotyrosine and found that either antipode had a toxicity identical with that of the racemic form. It will be recalled that Ferger and duVigneaud (1949) found a similar situation held for the optical forms of β -2-thienyl-DL-alanine. Subsequently, Armstrong and Lewis (1950) found 2- and 4-fluorotyrosine to be toxic for rats but to a lesser degree than the 3-isomer.

Actual proof that the toxicity of the halogenated analogues of tyrosine and phenylalanine was due to competitive inhibition came with the observations of Mitchell and Niemann (1947). They used *Neurospora* as the test organism and found 3-fluoro-DL-phenylalanine to be the most powerful agent with an inhibitor/metabolite ratio of 1:2. The optical antipodes of 3-fluorotyrosine were both active, but the L-form was approximately three times as powerful as the D. 2-Chloro-, 3-chloro-, 4-chloro-, 3-bromo-, 3-iodo-DL-phenylalanine and 3,5-difluoro-DL-tyrosine had inhibitor/metabolite ratios above 150. These investigators point out the similarity of the metabolite inhibitor relationship in this series with that seen between p-aminobenzoic acid and 3-fluoro-p-aminobenzoic acid and between acetate and fluoroacetate.

Martin and Moss (1949) tested a series of metabolite displacers of phenylalanine-tyrosine on the growth of *Pseudomonas aeruginosa* and

Table II

METABOLITE ANALOGUES OF PHENYLALANINE-TYROSINE PRODUCED BY
RING SUBSTITUTION

Compound	Action	Test System	Comments	Reference
3-Fluorotyrosine	+	Rat	Growth and toxicity	Boyer et al., 1947
	+	Neurospora	Growth	Armstrong & Elliott 1950
				Mitchell & New 1947
3-Fluorophenyl-alanine	+	Rat	Growth and toxicity	Boyer et al., 1947
	+	Neurospora	Growth	Mitchell & New 1947
3-Fluoro-5-iodo-tyrosine	+	Rat	Growth and toxicity	Boyer et al., 1947
3,5-Difluoro-tyrosine	+	Rat	Growth and toxicity	Boyer et al., 1947
	+	Neurospora	Growth	Mitchell & New 1947
2-, 3- and 4-Chlorophenyl-alanine	+	Neurospora	Growth	Mitchell & New 1947
3-Bromo- and 3-iodophenyl-alanine	+	Neurospora	Growth	Mitchell & New 1947
4-Fluorophenyl-alanine	+	<i>Pseudomonas aeruginosa</i>	Growth	Martin and M. 1949
	+	Rat	Growth	Armstrong & Elliott 1950
	+	Dopa decarboxylase	Inhibitor	Martin et al., 1949
3-Fluorophenyl-alanine	+	Rat	Growth	Armstrong & Elliott 1950
p-Aminophenyl-alanine	—	Hemolytic streptococci	Growth	Elliott et al., 1947
β-α-Amino-p-tolylalanine	+	Hemolytic streptococci	Growth	Elliott et al., 1947
p-Dimethyl-aminophenyl-alanine	—	Hemolytic streptococci	Growth	Elliott et al., 1947
3-Methyl-tyrosine	+	Dopa decarboxylase	Inhibition	Martin et al., 1949
	+	Dogs	Blood pressure	"
N-Methyl-p-fluorophenyl-alanine	—	"	"	Martin et al., 1949
	—	"	"	"
2-Methylphenyl-alanine	+	"	"	Martin et al., 1949
	—	"	"	"
Methyl-tyrosine	+	"	"	Martin et al., 1949
	+	"	"	"

p-fluorophenylalanine to be a competitive metabolic blocking agent. m-tyrosine, N-methyltyrosine, and 3-methyltyrosine were inactive. Additional members of this series listed in Table II were studied by Martin et al. (1948) using hemolytic streptococci as test organisms and by Martin et al. (1950) using dopa decarboxylase in vitro and also blood pressure in dogs. These results will be presented in the section on pharmacology but are listed in Table II.

Metabolite Analogues Produced by Alanine Side Chain Modification

Studies of molecular species which can be regarded as metabolite analogues of phenylalanine-tyrosine with side chain modification are few in number but ample to demonstrate the effectiveness of this approach.

In spite of the failure of Dakin (1909) to note any deleterious effect resulting from the administration of phenylserine to cats, Beerstecher and Shive (1946) investigated the displacement capacities of this molecule and found that it inhibited competitively with phenylalanine the growth of *Escherichia coli*, *Lactobacillus arabinosus*, and *Streptococcus faecalis* R. With other analogues of phenylalanine, it was reported that tryptophan and not tyrosine (Beerstecher and Shive, 1947) reversed the activity of phenylserine. This finding is in sharp contrast with the observation that tyrosine completely reverses the toxicity of β -2-phenylalanine, and is interpreted to indicate the blockage by phenylserine of the formation from

Table III

METABOLITE ANALOGUES OF PHENYLALANINE-TYROSINE PRODUCED BY SIDE CHAIN MODIFICATION; ALSO MISCELLANEOUS DISPLACEMENTS

Compound	Activity	Test System	Comments	Reference
Phenylserine Hydroxyphenylalanine	+	<i>Escherichia coli</i> <i>Lactobacillus arabinosus</i> <i>Streptococcus faecalis</i> R	Growth	Beerstecher & Shive, 1946
(p-Hydroxyphenyl)-aminoacetic acid	+		Antibiotic	Estermeyer and Kuhn, 1949
Hydroxyphenylpyruvic acid	+	Dopa decarboxylase	Activity	Martin et al., 1950
Acrylic acid	-	<i>Escherichia coli</i>	Growth	Martin, 1949
Maleic acid and derivatives	-	<i>Leuconostoc mesenteroides</i>	Growth	Furt and Harper, 1950
Acrylic acid	-	"	"	"
Maleic acid	-	"	"	"
Phenylacetic acid	+	<i>Escherichia coli</i> <i>Lactobacillus arabinosus</i>	Growth but non-competitive	Woolley, 1950

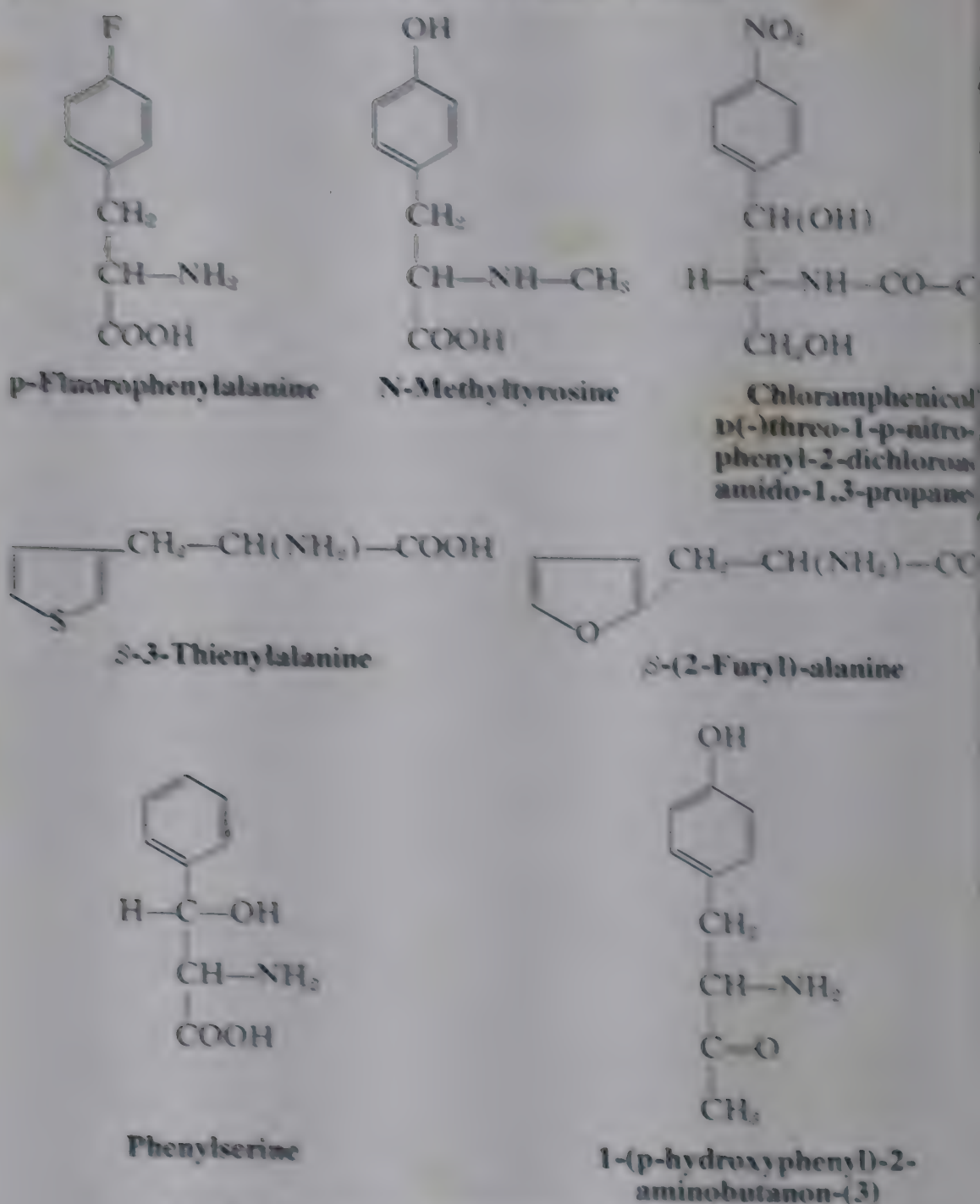


FIG. 2. Metabolite analogues of phenylalanine-tyrosine.

phenylalanine of an essential product other than tyrosine. It is interesting from the standpoint of possible chemotherapeutic application that the toxicity of this antimetabolite for animals has been confirmed (Arms and Lewis, 1950).

Other molecules of this general classification include 1-(p-hydroxyphenyl)-2-aminobutan-3-one which was synthesized and found to be antimetabolic by Erlenmeyer and Kahne (1949) and p-hydroxyphenylpyruvic acid which specifically inhibited the action of dopa decarboxylase (Nelson et al., 1950).

Miscellaneous Displacers

the category of miscellaneous displacers of phenylalanine can be added those primary tryptophan antimetabolites which are also reversed by phenylalanine. Indoleacrylic acid (Murray, 1949) is one of these analogues with no greater specificity for tryptophan than for phenylalanine. It is noted that *Escherichia coli* possesses a capacity for almost interchangeability of either amino acid in the systems specifically blocked by this metabolite. In this connection, it is interesting to note that 5-methyltryptophan is not an effective inhibitor of the action of dopa decarboxylase (Lin et al., 1950).

First and Harper (1950) reported the following compounds as even less active against phenylalanine using *Leuconostoc mesenteroides*: p-hydroxycinnamic acid, cinnamic acid, p-dimethylaminocinnamic acid, β -methylaminocinnamic acid, furylacrylic acid, and thienylacrylic acid. The action of α -aminophenylmethanesulfonic acid is not specific for phenylalanine as indicated by the failure of this amino acid to reverse its toxic action against the tubercle bacillus (Pope, 1949) or against dopa decarboxylase (Martin et al., 1950).

One of the complexities of the phenylalanine-tyrosine metabolite analogy series is contained in the capacity of tyrosine to inhibit the phenylalanine promoted growth of *Escherichia coli* (Beerstecher and Seive, 1941a). The action of phenylalanine is noncompetitive and is interpreted to indicate control by tyrosine over the biosynthesis of phenylalanine and related metabolites.

The most important contribution of recent date to the study of antagonism is the demonstration by Woolley (1950) that the antibiotic, Chloromycetin, acts by noncompetitive displacement of phenylalanine. He considered this antibiotic to be related to the amino acid structurally but to differ by presence of the nitro group, the dichloroacetyl moiety, the β -hydroxyl group, and the primary alcoholic unit. Using molecules changed at any one of these points and with combinations of two and three simultaneous modifications, he noted a reduction in competitive antagonism with increase in number of changes and a corresponding increase in the antibacterial, noncompetitive activity.

Enzymatic Aspects

The enzymatic concatenation involved in the metabolism of phenylalanine and tyrosine is extremely complex but it seems probable that any one can be blocked by metabolite analogues. The first to consider would be blockage of enzymatic mechanisms involved in protein synthesis. In protein depleted animals, Dittmer et al. (1950) noted inhibition of weight gain by β -3-thienylalanine, which observation can be interpreted

to indicate interference with the activity of enzymes involved in protein synthesis.

Another phase is the oxidative enzymatic systems by which phenylalanine is oxidized to tyrosine, tyrosine to dopa, and dopa to melanin. The first step is inhibited by β -2-thienylalanine when *Escherichia coli* is the test system (Beerstecher and Shive, 1947); the second step and the third which is that of conversion of dihydroxyphenylalanine to melanin have been investigated and many inhibitors of this reaction are known. N-Acetyltyrosine, N-formyltyrosine, and 3-fluorotyrosine (Lerner and Fitzpatrick, 1950) are effective inhibitors of the tyrosine-tyrosinase reaction and of the dopa-tyrosinase reactions in vitro. These substances apparently act as competitive analogues of the natural substrates for active centers on tyrosinase. Observations on the inhibition of melanin formation in mice, rats, and cats by hydroquinone (Oettel, 1936; Martin and Ansbacher, 1941) probably represent in vivo blockage of the oxidative mechanisms. Reducing agents tend to prevent melanin formation both in vivo and in vitro. Epinephrine is subject to the action of tyrosinase, and its oxidation product is a melanin. Martin et al (1942) reported inhibition of epinephrine oxidation by aromatic amines and aminobenzoic acids. Displacement was not demonstrated to be the basis of the results obtained but in one instance, namely with ephedrine the activity is doubtless competitive in nature. It has long been established that the pharmacological power of ephedrine represents a reflection of its displacement of epinephrine from an amine oxidase (Gaddum and Kwiatkowski, 1938). Tyrosinase function can also be blocked by an inorganic material such as molybdenum (Muir, 1941) which displaces the copper of the enzyme, or by a chelating agent such as phenylthiocarbamide (Bernheim and Bernheim, 1942), an agent interfering with the normal functioning of copper.

Another enzymatic system of great importance to pharmacological aspects of tyrosine and phenylalanine metabolism is that of dopa decarboxylase. This will be considered in the next section because it represents the key to the formation in vivo of pressor amines.

Tyrosine decarboxylase occurs in bacteria but is not known to occur in animals. Recently, Frieden and Dittmer (1950) studied the effect of a series of antagonists on this enzyme and reported that the enzyme decarboxylated certain molecules tested, including: β -2- and β -3-di-thienylalanine, 3,5-difluorotyrosine, and 3-amino-L-tyrosine. The thienyl compounds did not reduce the rate at which tyrosine was decarboxylated. 3,5-Dibromo-L-tyrosine, 3,5-diiodo-L-tyrosine, 3-nitro-L-tyrosine, p-hydroxyphenyl-DL-glycine, and p-tolyl-DL-alanine did not serve as substrates.

The coenzyme for tyrosine decarboxylase is phosphorylated pyridoxal, and it was therefore of interest that while desoxypyridoxine failed, phosphorylated desoxypyridoxine did inhibit (Beiler and Martin, 1947). The related observation that tyrosine is not as toxic to the pyridoxine deficient

at (Martin, 1946) indicates the possibility that this B-complex factor is involved in tyrosine metabolism in the animal as well as in bacterial systems. Tyrosine decarboxylase is definitely not present in the animal but some similar enzyme is probably involved.

In any event, chemical agents for controlling the formation of tyramine by bacteria in the gastrointestinal tract might well prove valuable if the research of the future demonstrates the involvement of tyramine and similar agents in the diseases of aging.

Chemotherapy and Pharmacology

The formation of pressor amines involves the interplay of many seemingly divergent factors. One would be substrate concentration and would depend on rate of supply and rate of destruction of phenylalanine and tyrosine. Excess tyrosine in the diet is known to produce hypertension (Martin, 1943) and the toxicity of this amino acid is increased in animals deficient in riboflavin (Martin, 1947). The explanation of the first observation is clear and in the second instance the fact that lower concentrations of amino acid oxidase, which is a riboflavin-containing enzyme, would naturally result from a deficiency state, determines the lower rate of oxidative deamination of tyrosine with resultant greater substrate concentration.

Another factor in the formation in vivo of pressor amines would be the rate of conversion of tyrosine to dihydroxyphenylalanine, because the decarboxylase does not act on tyrosine. This aspect has not as yet received adequate study.

The next phase of importance in pressor amine formation is the activity of dopa decarboxylase. If the biological conversion of dopa to pressor amine could be inhibited, the logical result would be a lowering of blood pressure in the normal animal and possibly in the hypertensive. One approach would be through a study of cofactors for the enzyme. Desoxypyridoxine phosphate did not inhibit the decarboxylase, while folic acid analogues did (Martin and Beiler, 1947, 1948). These same folic acid antagonists produced a marked drop in blood pressure in the intact animal (Martin et al., 1947, 1948), which can be interpreted as a manifestation of in vivo inhibition of the decarboxylation of dihydroxyphenylalanine.

Another method of approach to this problem should be by the inhibition of decarboxylation through the displacement of dopa from the enzyme surface by the use of structural analogues. A series of such displacers has been reported effective in the inhibition of dopa decarboxylase and the lowering of blood pressure in the intact animal (Martin et al., 1950). Table IV shows the scope of this work.

The final mechanism involved would be the rate of destruction of pressor amines by amine oxidases. This phase has been discussed in the preceding section under Enzymatic Aspects.

Table IV

EFFECT OF TYROSINE AND DIHYDROXYPHENYLALANINE ANALOGUES ON THE ACTIVITY OF DOPA DECARBOXYLASE AND ON THE BLOOD PRESSURE OF THE INTACT ANIMAL*

Substance	Concentration	Per Cent Inhibition Decarboxylase	Dose Kg. Wt.	Effect on Blood Pressure
Suramine	300 γ /cc. 30 γ /cc.	65% 40%	10 mg./kg.	— 30 mm. 30 min.
3-Methyltyrosine	1.2 mg./cc.	45%	10 mg./kg. 25 mg./kg.	No effect — 90 mm. 10 min.
N-Methyl-p-fluorophenylalanine	1.2 mg./cc.	No effect	75 mg./kg.	No effect
p-Fluorophenylalanine	1.2 mg./cc.	25%	25 mg./kg. 50 mg./kg.	— 30 mm. 4 hrs. — 70 mm. 9 hrs.
p-Methylphenylalanine	1.2 mg./cc.	35%	20 mg./kg.	No effect
p-Chlorophenylalanine	1.2 mg./cc.	50%	68 mg./kg.	No effect
N-Methyltyrosine	2 mg./cc.	50%	5 mg./kg.	No effect
N-Methyl dihydroxyphenylalanine	2 mg./cc.	50%	5 mg./kg.	+ 10 mm. 2 min.
α -Aminophenylethane sulfonic acid	1.2 mg./cc.	100% Nonspecific	5 mg./kg.	No effect
α -Aminophenylmethane sulfonic acid	1.2 mg./cc.	100% Nonspecific	20 mg./kg.	No effect
4-Imidazyl aminomethyl sulfonic acid	1.2 mg./cc.	30% Nonspecific	50 mg./kg.	No effect
o-Fluorobenzoic acid	1.2 mg./cc.	40% Nonspecific	20 mg./kg.	No effect
p-Hydroxyphenylpyruvic acid	1.2 mg./cc.	40%	20 mg./kg.	— 24 mm. 10 min.
β -2-Thienylalanine	1.2 mg./cc. 2.5 mg./cc.	No effect 40%		
Dihydroxyphenylalanine			50 mg./kg.	+ 110 mm. 60 min.
p-Fluorophenylethylamine HCl	1.2 mg./cc.	No effect	3 mg./kg.	+ 100 mm. 10 min.
α -Amino-p-fluorophenylbutyric acid	1.2 mg./cc.	No effect (insoluble)	5 mg./kg.	No effect
5-Methyltryptophan	1.2 mg./cc.	No effect	10 mg./kg.	— 20 mm. 2 min.
Methionine sulfoxide	1.2 mg./cc.	No effect	50 mg./kg.	No effect
Benadryl	1.2 mg./cc.	No effect	2 mg./kg.	— 30 mm. 3 min.
2-Isopropyl-5-methylphenoxyethyl diethylamine HCl	1.2 mg./cc.	No effect	5 mg./kg.	— 90 mm. 5 min.
Phenylalanine	1.2 mg./cc.	65%	10 mg./kg.	No effect
Phenylserine	1.2 mg./cc.	15%	200 mg./kg.	No effect
Methoxytyrosine, O-methyltyrosine	1.2 mg. cc.	20%	5 mg./kg.	— 30 mm. 1 min.
Diiodotyrosine	1.2 mg. cc.	20%	5 mg./kg.	No effect
2,5-Dimethyl- β -phenylalanine	1.2 mg./cc.	20%	20 mg./kg.	-- 20 mm. 2 min.

* From *Exper. Med. & Surg.*, 8, 5, 1950.

Another phenomenon correlated with the toxicity of tyrosine is the modifying effect of other amino acids. Sullivan and his associates (1932) noted the counteraction by cystine of the deleterious effects of tyrosine which observation was confirmed by Martin (1947) who also found glycine effective.

It seems probable that one approach to the problem of hypertension would involve reduction of intake of phenylalanine-tyrosine, provision of adequate riboflavin to insure ample amino acid oxidase, inhibition of the action of dopa decarboxylase through both cofactor and substrate displacement, and finally optimal function of amine oxidase mechanisms. This approach has not yet been made.

Fluorinated derivatives of phenylalanine and tyrosine have been tested as agents for the control of basal metabolic rate but they were not possessed of an adequate therapeutic ratio for clinical application (Boyer et al., 1941). 3-Fluorotyrosine failed to alter the growth of an "ascites" tumor in the mouse (Niedner, 1941).

Applications of phenylalanine-tyrosine metabolite analogues in chemotherapy have not been extensive. The indications are that practical applications are likely to meet with success. The action of 2- and 3-pyridylalanine in preventing the adsorption of the phage particle (Anderson, 1946) and the antibacterial efficacy of various analogues against the tubercle bacillus (Pope, 1949), *Escherichia coli* (Dittmer et al., 1946), and *Pseudomonas aeruginosa* (Martin and Moss, 1949) strongly indicate the logic of further studies in this field.

Recapitulation

As consideration of the design of metabolite analogues is extended, it becomes clear that with ring and side chain containing molecules effective agents can be formed by ring modification, ring substitution, or side chain alteration. In the case of the phenylalanine-tyrosine group, β -2-thienylalanine is typical of the first, p-fluorophenylalanine of the second, and phenylserine of the third type.

Virtually every enzyme involved in the metabolism of these amino acids has been found susceptible to the action of structural inhibitors. The enzymatic mechanisms of protein synthesis are prevented by β -3-thienylalanine; the action of tyrosinase is blocked by N-acetyltyrosine; the conversion of phenylalanine to tyrosine is stopped by β -2-thienylalanine; and the activity of dopa decarboxylase is reduced by p-fluorophenylalanine.

The chemotherapeutic and pharmacological applications of displacers of this category have been few but the potentialities are indicated by the capacity of the pyridylalanine in preventing virus adsorption, and by the efficacy of p-fluorophenylalanine and other similar molecules in blood pressure reduction. In this latter consideration, the interrelated phenomena

point up clearly the necessity for consideration of the overall pattern rather than restriction of approach to a single phase. Here the goal is reduced concentrations of pressor amines and this would best be done by an approach designed to reduce intake of precursors, minimize decarboxylase substrate by optimal amino acid oxidase activity, retard phenylalanine oxidation to tyrosine and dopa, reduce the activity of the decarboxylase through substrate or cofactor displacement, and finally gain optimal activity of amine oxidase.

The lack of specificity of displacement might be considered a factor against the application of these agents but it is encouraging to consider the nontoxicity of phenylserine to animals with its powerful antibacterial action. This establishes the probability that certain phases of phenylalanine-tyrosine metabolism essential to bacteria are not vital to animals. It is this differential requirement for natural metabolite which forms the basis for the general application of metabolite analogues in chemotherapy and pharmacology.

The observation that Chloromycetin is a noncompetitive displacer of phenylalanine opens new worlds of research in the field encompassed by the displacement concept. It indicates that strictly competitive inhibitors may not form the best practically applicable agents and the falsity of the general belief that multiple simultaneous modifications in a given structure result in absence of activity. The importance of emphasis on the distinction between competitive and noncompetitive antimetabolites is established. A new class of multiple modification metabolite analogues is now revealed as a fruitful field. The permutations and modifications possible using any one metabolite as model are enormous.

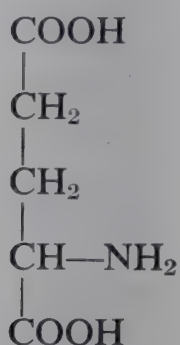
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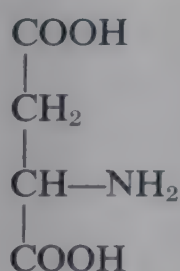
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Chapter 8

GLUTAMIC AND ASPARTIC ACID ANALOGUES



Glutamic acid



Aspartic acid

1. Methionine Sulfoxide and Related Molecules as Glutamic Acid Displacers.
2. All Other Metabolite Analogues of Glutamic Acid.
3. Aspartic Acid Metabolite Analogues.
4. Metabolite Analogues of Glutamic Acid Peptides.
5. Metabolite Interrelationships of Aspartic and Glutamic Acids.
6. Recapitulation.

Although not one of the essential amino acids, glutamic acid is nevertheless of great importance as it is primarily involved in transamination reactions. The enzyme catalyzing this chemical mechanism is transaminase, which occurs in practically all animal tissue, including heart muscle, brain, kidney, testicle, etc. It is present also in plants and in microorganisms. The following reaction is typical of transamination:



In this reaction, aspartic acid can replace glutamic acid as follows:



α -Ketobutyric and oxalacetic acids accept the amino group of glutamic acid and conversely some 16 different amino acids produce glutamic acid from α -ketoglutaric acid in the presence of muscle tissue. The rate of this reaction, high in animals, is still higher in plants and microorganisms. Ammonia can serve as the source of the amino group, a fact tending to emphasize the key position of these dicarboxylic acids in protein metabolism. Transamination reactions studied by isotope technics clearly are continuous processes and play an essential role in the rapid metabolism of cell

proteins. Phosphorylated pyridoxal is a coenzyme for transaminase and also for glutamic acid decarboxylase. The amino group of glutamic acid is transferred first to pyridoxal forming pyridoxamine phosphate which in turn transfers the amino group to the keto acid. The amino acid oxidases do not deaminate either of the dicarboxylic amino acids.

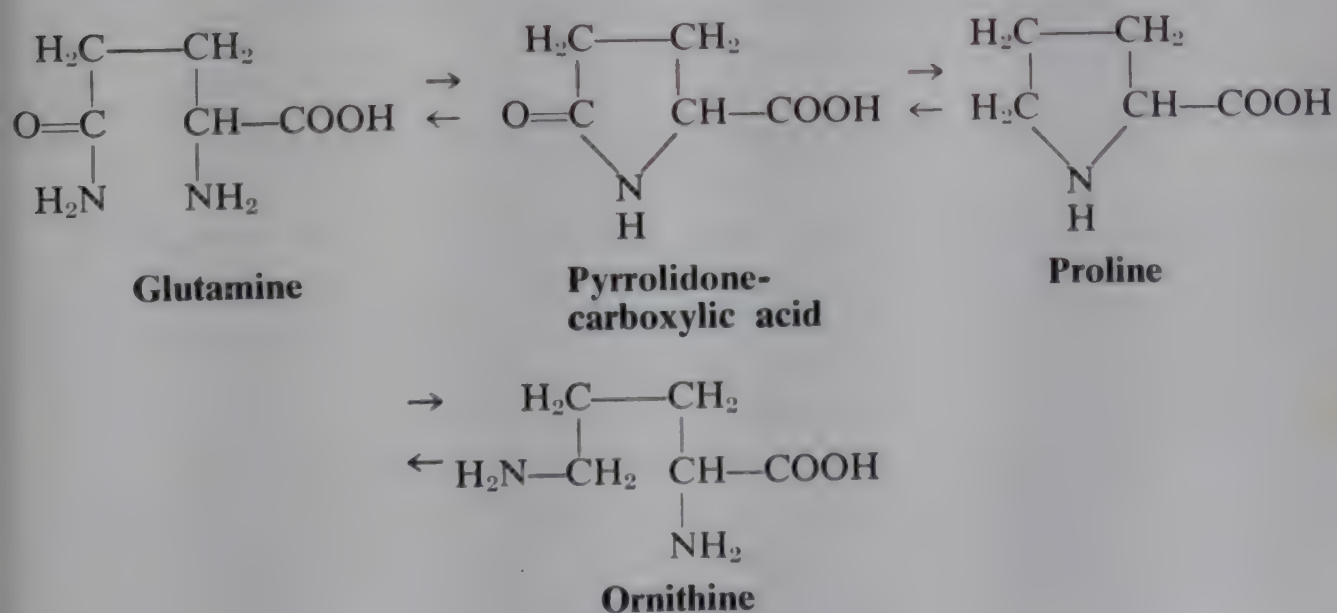
Both glutamic and aspartic acids on oxidative deamination form keto acids, components of the tricarboxylic cycle of carbohydrate metabolism. From carbohydrate via the keto acids and amination reactions both glutamic and aspartic acids are formed, and from the glutamic come glycine, serine, cysteine, cystine, proline, hydroxyproline, ornithine, and arginine. The γ -carboxyl of glutamic acid is probably important in bridges between peptide chains in protein structure.

Folic acid is a peptide of glutamic acid and occurs as conjugates with two and as many as seven glutamic acid residues. Glutathione is another such peptide molecule, being a tripeptide (glutamylcysteinylglycine) and in it occur all of the amino acids known to participate in detoxication reactions. The significance of this has not been determined. L-Serylglucyl-L-glutamic acid possesses streptogenin activity. Finally, another glutamic acid peptide—glutamyl polypeptide—has been isolated from *Bacillus subtilis*.

D-Glutamic acid, the unnatural form, is found in the capsular substance of *Bacillus anthracis* and in the antibiotic agents, gramicidin and tyrocidin.

During amination the amino groups are partially transferred from the α -carbon of the amino acid to the γ -carbon of glutamic acid with resultant formation of glutamine. Adenosinetriphosphate is involved in the reaction which closely parallels the metabolic concatenation of sulfanilamide acetylation.

Another metabolic sequence is that of D-glutamic acid to pyrrolidone-carboxylic acid which on reduction forms proline. Proline in turn forms ornithine in accordance with the following:



Aspartic acid acts as the ammonia donor in the conversion of citrulline to arginine in the liver. Arginine is a sometime-essential amino acid and inhibition of its formation might prove critical in some aspartic acid metabolite analogue experiments. Furthermore, the formation of urea from arginine must be considered in such research, as both glutamic acid and aspartic acid are involved. In connection with the use of glutamic acid in epileptics and mental defectives, it is interesting to note that it is the only amino acid capable of replacing glucose in the maintenance of the respiration of brain cortex slices. The favorable effect of glutamic acid administered in mental disease, a controversial finding, is attributed to the activation of cholineacetylase. Conversely, glutamic acid inhibits brain and kidney glutaminase which catalyzes the hydrolysis of the amide, glutamine.

Glutamic and aspartic acids are nonessential to higher animals but certain bacteria require them for optimal growth. The prime function in bacterial systems, as in animal, is that of transamination reactions catalyzed by aminopherases.

Aspartic acid can be formed from fumaric acid by bacteria. Nitrogen-fixing bacteria form the amino acid from oximinosuccinic acid. Biotin is probably involved in the synthesis of aspartic acid by bacteria; however, transamination does not seem to be the limiting factor in biotin deficiency. The breakdown products of aspartic acid by microorganisms include malic acid, succinic acid, fumaric acid, β -alanine, propionic acid, acetic acid, n-propyl alcohol, and ammonia. For glutamic acid, the list includes α -ketoglutaric acid, glutaric acid, and ammonia. Glutamic acid is required by *Rhodospirillum rubrum*, some strains of *Eberthella typhosa*, certain streptococci, *Corynebacterium diphtheriae*, lactic acid bacteria, and *Neisseria intracellularis*. Glutamine under certain conditions stimulates growth of or is required by hemolytic streptococci and pneumococci, some strains of *Bacillus anthracis*, lactic acid bacteria, and gonococci. Glutamic acid at higher levels usually will replace glutamine. Some lactic acid bacteria, for example, *Lactobacillus delbrückii*, require aspartic acid.

Methionine Sulfoxide and Related Molecules as Glutamic Acid Displacers

The first studies of metabolite analogues of glutamic acid were motivated by observations on the efficacy of L-glutamic acid in the treatment of petit mal epilepsy (Price et al., 1943; Waelsch and Price, 1944) and mental defectives (Albert et al., 1946). Using *Lactobacillus arabinosus* as the test organism, Waelsch et al. (1946) found that the sulfoxides of DL-methionine and DL-benzyl-homocysteine were effective antimetabolites. The sulfonyl derivative of methionine exerted a nonspecific effect. In the same nonspecific manner, cysteine tended to reverse the action of the sulfoxide, probably by reducing the molecule to the original sulfur ether form.

Table I
METABOLITE ANALOGUES OF GLUTAMIC ACID

Compound	Activity	Test System	Comment	Reference
Methionine sulfoxide	+	<i>Lactobacillus arabinosus</i>	Glutamic acid→ glutamine	Waelsch et al., 1946
Benzylhomo-cysteine sulfoxide	+	"	"	Waelsch et al., 1946
Ethionine sulfoxide	—	"	"	Waelsch et al., 1946
n-Butylhomo-cysteine sulfoxide	+	"	"	Borek & Waelsch, 1947
n-Propylhomo-cysteine sulfoxide	—	"	"	Waelsch et al., 1946
n-Amylhomo-cysteine sulfoxide	+	"	"	Borek & Waelsch, 1947
n-Hexylhomo-cysteine sulfoxide	+	"	"	"
n-Laurylhomo-cysteine sulfoxide	+	"	"	"
α-Aminoadipic acid	—	"	"	Waelsch et al., 1946
Aminomalonic acid	—	"	"	"
i-β-Phenylglutamic acid	—	"	"	"
DL-"para"-Hydroxy-aspartic acid	+	<i>Escherichia coli</i>	Glutamic converted to aspartic acid	Shive & Macow, 1946
α-Aminopimelic acid	—	<i>Lactobacillus arabinosus</i>	Glutamic acid→ glutamine	Borek & Waelsch, 1949
β-Hydroxyglutamic acid	+	"	"	"
DL-N-(γ-Glutamyl)-ethylamine	+	<i>Staphylococcus aureus</i>	Growth	Lichtenstein & Grossowicz, 1947
L-N-(γ-Glutamyl)-n-butylamine	—	"	"	Lichtenstein & Grossowicz, 1947
L-N-(γ-Glutamyl)-ethanolamine	+	"	"	"
DL-N-(γ-Glutamyl)-ethanolamine	+	"	"	"
α-Amino-γ-propyl-succinic acid	—	<i>Streptococcus faecalis</i>	Growth	Roper & McIlwain, 1948
α-Amino-β-butyl-succinic acid	—	"	"	"
α-Amino-β-octyl-succinic acid	—	"	"	"
α-Aminoundecane-α, λ-dicarboxylic acid	—	"	"	"
α-Amino-α-methyl-valeric acid	—	"	"	"

Table 1—(Continued)

Compound	Ac-tivity	Test System	Comment	Reference
α -Amino- β -furyl-propionic acid	—	<i>Streptococcus faecalis</i>	Growth	Roper & McIlwain, 1948
N-(γ -glutamyl)-hydroxylamine	+	"	"	"
γ -Glutamylhydrazine	+	"	"	"
Acetone derivative γ -Glutamylhydrazine	+	"	"	"
γ -Ethylglutamate	—	"	"	"

Methyl and benzyl derivatives were highly active but the corresponding ethyl compound, sulfoxide or the sulfone, was inactive. This is a striking example of specificity for which the authors offer no explanation. Neither of the active glutamic acid antagonists was effective against glutamine, which apparently indicates that the sulfoxides prevent the amidation of glutamic acid. This interpretation finds support in the observations of Elliott and Gale (1948) who used a *Staphylococcus aureus* glutamine synthesizing system and checked the formation of a hydroxamic acid in the presence of glutamic acid, adenosinetriphosphate, and hydroxylamine. The enzyme was competitively inhibited by methionine sulfoxide.

Other phases of metabolism may also be blocked by the sulfoxides; one such is suggested by the failure of ketoglutaric acid to reverse the toxicity of these antimetabolites (Waelsch et al., 1946), indicating inhibition of the amination of the ketoglutaric acid or a general inhibition of transamination reactions. Another point tending to substantiate this view is the fact that glutamine does not completely reverse the sulfoxides (Borek and Waelsch, 1949).

Reversal of the antimetabolites by L-glutamic acid was observed, and of particular interest is the discovery that glutathione was almost as effective. Neither methionine nor aspartic acid was active (Borek et al., 1946).

In an attempt to elucidate the failure of the sulfoxide of ethionine as an antimetabolite, Borek and Waelsch (1947) prepared the corresponding sulfoxides and sulfones of n-propyl-, n-butyl-, n-amyl-, n-hexyl-, and n-laurylhomocysteine. The propyl sulfoxide was as inactive as the ethyl analogue but, starting with the butyl homologue, increasing inhibition of bacterial growth occurred as the chain length increased. The inhibition observed with these compounds was only partially reversed by glutamine or glutamic acid.

This same paper reports that the dissociation constants of the sulfoxides

of methionine and ethionine were identical, a finding which excludes dissociation constants as the basis of the observed difference in glutamic acid displacement capacity.

Following the resolution of the diastereoisomeric sulfoxides of L-methionine by Lavine (1947), Waelsch and Borek (1947) and Borek and Waelsch (1947a) studied the biological activities of these compounds. The mixture of diastereoisomers derived from L-methionine was about seven times as active as those derived from D-methionine. The diastereoisomers derived from L-methionine differed in their antimetabolite capacity by a ratio of 2.5. These authors interpret this as indicating that the carbon configuration is more important to enzymatic activity than the sulfur.

All Other Metabolite Analogues of Glutamic Acid

During the course of their work with methionine sulfoxide, Waelsch et al. (1946) found that DL- α -aminoadipic acid, aminomalonic acid and i- β -phenylglutamic acid were inactive as displacing agents for glutamic acid. In the same year, Shive and Macow reported the efficacy of DL-"para"-hydroxyaspartic acid as a competitive inhibitor of aspartic acid. Glutamic acid reversed the hydroxyaspartic acid over a wide range of concentrations and was thought to act as a precursor of aspartic acid in the system studied.

β -Hydroxyglutamic acid is a potent antimetabolite for glutamic acid (antibacterial index of 30), functioning in a manner identical with that of methionine sulfoxide (Borek and Waelsch, 1949). By contrast, α -aminopimelic acid was ineffective.

Following Woolley's (1946) proposal, Lichtenstein and Grossowicz (1947) prepared a number of glutamic acid analogues in which the γ -carboxyl was modified. These included DL-N-(γ -glutamyl)-ethylamine, DL-N-(γ -glutamyl)-ethanolamine, L-N-(γ -glutamyl)-ethanolamine, and L-N-(γ -glutamyl)-n-butylamine of which all but the last markedly inhibited the growth of *Staphylococcus aureus*, an effect reversed by glutamic acid. The inhibitory action of these γ -glutamylamines is delayed for 24 to 27 hours, the explanation being that although there is present in the medium a concentration of glutamic acid adequate to overcome the inhibitor initially, it is consumed as growth proceeds, and the inhibitory effect of the antimetabolite is observed.

A point of great importance in the work of Lichtenstein and Grossowicz is their observation that the inhibition induced by the alkyl substituted amides of glutamic acid was not reversed by glutamine, an indication that free glutamic acid is essential for the normal growth of *Staphylococcus aureus* and that these antimetabolites block the deamidation of the amide, glutamine.

Of a series of 14 compounds tested by Roper and McIlwain (1948), only four were capable of inhibiting the growth of streptococci. The negative

molecules as well as those found active are listed in Table I. The series included methionine sulfoxide and sulfone—both of which were found active as previously reported (Waelsch et al., 1946). The new molecules not of prior record as antimetabolites for glutamic acid included N-(γ -glutamyl)hydroxylamine, γ -glutamylhydrazine, and the corresponding acetone derivative. These authors indicate that the basis of action of these compounds, excepting the hydroxamic acid, is related to the metabolism of glutamic acid or glutamine. Actually it would seem that here, as with the molecules tested by Lichtenstein and Grossowicz, the compounds of the type of γ -glutamylhydrazine prevented deamidation of glutamine (the compound stopped ammonia formation from glutamine) and that glutamic acid must be essential to glycolysis in the test bacteria.

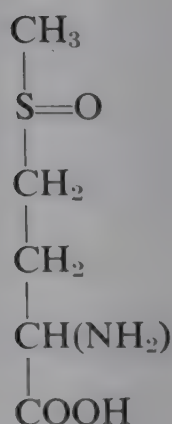
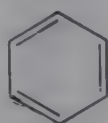
Subsequently, this same group (McIlwain et al., 1948) reported a detailed study of the stimulating effect of glutamine on glycolysis in suspensions of β -hemolytic streptococci and of the inhibition of this reaction by γ -glutamylhydrazine. They emphasize that glutamic acid and ammonia do not constitute the sole factors necessary for reversal of the inhibitory action of glutamylhydrazine. It was made apparent in their studies that both the synthesis and breakdown of glutamine is essential to the normal growth of streptococci.

The existence of both competitive and noncompetitive inhibitors of any given enzymatic reaction is a foregone conclusion. In the case of the glutamine synthesizing enzymatic system in *Staphylococcus aureus*, it has been stated that methionine sulfoxide is a competitive inhibitor and now crystal violet is added as a noncompetitive inhibitor (Elliott and Gale, 1948).

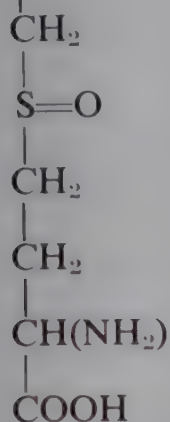
Aspartic Acid Metabolite Analogues

One of the most concise demonstrations of the complexity of the inter-related systems involved in any metabolite analogue study is the report of Ravel and Shive (1946) in which cysteic acid is demonstrated to be a competitive antagonist of aspartic acid. *Escherichia coli* was used as the test system in their studies and they found that not only aspartic acid but β -alanine and pantothenic acid nullified the action of the antimetabolite. The interpretation of these findings was that cysteic acid prevented the decarboxylation of aspartic acid with resultant formation of β -alanine, in its turn incorporated into the pantothenic acid molecule.

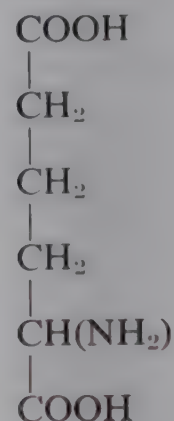
Glutamic acid was more powerful than aspartic acid as a reversing agent for the toxicity of cysteic acid. Certain correlated studies were made in an attempt to ascertain the basis of this reversal; α -ketoglutaric acid was as effective as aspartic acid in reversing the toxicity, and mixtures of α -ketoglutaric and aspartic acids equaled or exceeded the potency of glutamic acid.



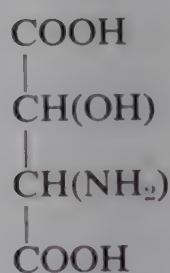
Methionine sulfoxide
(*i*- γ -methylsulfinyl- α -aminobutyric acid)



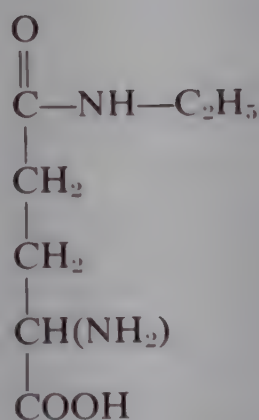
Benzylhomocysteine sulfoxide



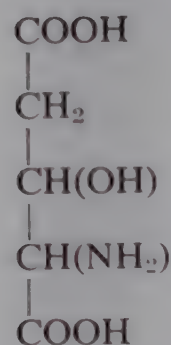
α -Aminoadipic acid



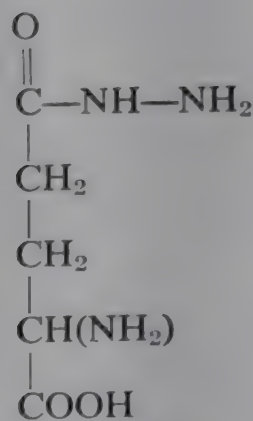
"para"-Hydroxyaspartic acid



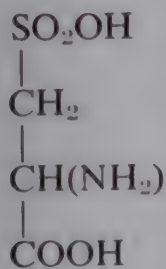
DL-N-(γ -Glutamyl)-ethylamine



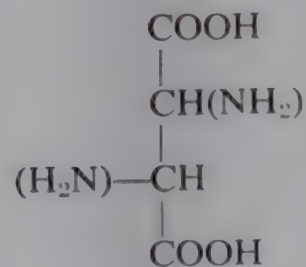
β -Hydroxyglutamic acid



γ -Glutamylhydrazine



Cysteic acid



Meso-Diaminosuccinic acid

FIG. 1. Metabolite analogues of glutamic and aspartic acids.

Attempts at an explanation of these observations bring into the picture similar studies carried out by Shive and Macow (1946) with DL-"para"-hydroxyaspartic acid (the isomer converted to DL-tartaric acid by treatment with nitrous acid). This compound inhibited competitively some functions of aspartic acid in *Escherichia coli* (molar inhibition ratio 10 to 15). Reversal of the action of the antimetabolite was effected by aspartic acid, glutamic acid, and in the presence of low levels of hydroxyaspartic acid, pantothenic acid, β -alanine, and asparagine were also effective.

Table II
METABOLITE ANALOGUES OF ASPARTIC ACID

Compound	Ac- tivity	Test System	Comment	Reference
Cysteic acid	+	<i>Escherichia coli</i> Lactobacilli	Growth	Ravel & Shive, 1946
DL-"para"-Hydroxy- aspartic acid	+	<i>Escherichia coli</i>	Growth	Shive & Macow, 1946
Meso-Diamino succinic acid	+	<i>Escherichia coli</i>	Growth	"
β -Aspartylhydrazine	+	<i>Streptococcus faecalis</i>	"	Roper & McIlwain, 1948
N-(β -Aspartyl) hydroxylamine	+	"	"	"

One point seems clear from these observations and it is that cysteic acid inhibits only the biosynthetic processes for pantothenic acid formation, while hydroxyaspartic acid inhibits this process in at least one additional aspartic acid metabolic channel. This would account for the complete reversal of the action of cysteic acid by β -alanine and pantothenic acid, and the limited capacity of these agents in the reversal of hydroxyaspartic acid.

A combination of α -ketoglutaric acid and aspartic acid possesses a reversing action for cysteic acid equal to or exceeding that of glutamic acid (glutamic having in turn three times the power of aspartic). Now with hydroxyaspartic acid, the enhanced effect of glutamic acid over aspartic acid does not occur. Furthermore, α -ketoglutaric acid has a relatively low activity in reversing hydroxyaspartic as compared to cysteic acid. This suggests that hydroxyaspartic inhibits transamination reactions as well as the enzymatic decarboxylation of aspartic acid to β -alanine.

Inasmuch as the combined ketoglutaric and aspartic acids equal or surpass glutamic acid, it is reasonable to assume that the limiting factors in the reactions inhibited are glutamic acid and ketoglutaric rather than aspartic acid when cysteic acid is the antimetabolite. There seems little reason to regard cysteic acid as primarily an antimetabolite effective against aspartic acid; it appears rather to be effective against glutamic acid primarily and

aspartic secondarily with the restriction of the involvement of these dicarboxylic amino acids in pantothenic acid synthesis. In other words, it seems probable that glutamic or ketoglutaric acids act as cofactors for the enzymatic decarboxylation of aspartic acid.

Cysteic acid inhibits the growth of *Lactobacillus casei*, *Lactobacillus arabinosus*, and *Leuconostoc mesenteroides*; hydroxyaspartic acid is effective against *Leuconostoc mesenteroides*. With these bacteria, at least in the case of cysteic acid, reversal of action is effected by aspartic acid but not by glutamic. β -Alanine had no effect on the toxicity of cysteic acid for *Lactobacillus arabinosus*, indicating that in this instance the biosynthetic channels leading to pantothenic acid are not primarily involved.

Meso-Diaminosuccinic acid was also found by Shive and Macow (1946) to be an effective competitive metabolite antagonist for aspartic acid when *Escherichia coli* was the test organism.

Asparagine partially reversed the toxic action of both hydroxyaspartic (Shive and Macow) and cysteic acids (Ravel and Shive), indication that these antimetabolites do not affect deamidation primarily, unlike other agents such as alkyl substituted amides of glutamic acid (Lichtenstein and Grossowicz).

β -Aspartylhydrazine was prepared and tested by Roper and McIlwain (1948). It inhibited the growth of *Streptococcus faecalis* strains and in some instances the toxic action was diminished by asparagine or glutamine. N-(β -Aspartyl) hydroxylamine was also an active growth inhibitor.

Metabolite Analogues of Glutamic Acid Peptides

In the introduction to this section, three peptides of glutamic acid were listed as of major metabolic significance: glutathione, folic acid, and strepogenin.

The first of these, glutathione, acts as a coenzyme for glyoxalase; the effects of several analogues were tested in this system by Behrens (1941). Isoglutathione (α -glutamylcysteinylglycine) which differs from glutathione only in that the glutamic acid residue is attached through the alpha-carboxyl instead of the gamma, and asparthione (β -aspartylcysteinylglycine) which is the aspartic acid analogue of glutathione, were both found capable of acting as coenzymes and exerted no toxic action as tested.

This probably indicates that the functional unit of glutathione is the sulfhydryl of the cysteine, with the glutamyl residue of minor importance.

The second peptide of glutamic acid is folic acid, which has a multiplicity of functions. In this case, modification in the glutamic acid moiety produces a displacer or eliminates activity. For example, pteroylaspartic acid (Hutchings et al., 1947) and the glycine and methionine sulfoxide analogues of folic acid (Martin et al., 1948, Woolley and Pringle, 1948) were effective antimetabolites.

The third type of peptide is strepogenin, the activity of which is possessed to a limited degree by L-serylglycyl-L-glutamic acid and similar molecules. Serylglycylaspartic acid (Woolley, 1948) was found inhibitory to the growth promoting action of strepogenin. At concentrations higher than those exhibiting an antagonistic action, the aspartic acid peptide actually manifested some growth promoting action.

Metabolite Interrelationships of Aspartic and Glutamic Acids

Competitive inhibition among the various amino acids is general; therefore, it is not surprising that a biological antagonism exists between aspartic and glutamic acids. Mulinos et al. (1950) found that imbalance resulting in an excess of either acid was inhibitory to acid production by *Streptococcus faecalis*. The optimal aspartic-glutamic ratio was 1.6.

Biotin substitutes for aspartic acid in the metabolism of *Streptococcus faecalis*; conversely aspartic acid reverses the antimetabolite action of biotin displacers. This interrelationship suggests the importance of a study of combinations of biotin and aspartic acid metabolite analogues.

The biological antagonism of naturally occurring metabolites is seen again in the ability of glutamine to inhibit the growth promoting effect of glutamic acid for *Staphylococcus aureus* (Grossowicz, 1948). This action of glutamine was reported reversed by pteroylglutamic acid, glutathione and glutamic acid, but the observation has been challenged by Hughes (1948) who indicated that the zinc content of the glutamine was responsible for the inhibitory effect.

Recapitulation

The possibility arises that glutamic acid antimetabolites might prevent formation or functioning of any physiologically important glutamic acid containing peptide (i.e., glutathione, strepogenin, pteroylglutamic acid). No studies of the action of such metabolite analogues have been made in vivo but it is probable that a degree of specificity related to concentration will be observed. Considering the possible points of inhibitory action, such as transamination, protein synthesis, peptide formation, amidation, deamidation, conversion to any one of an entire sequence of amino acids, vitamin precursor, etc., it will be most challenging to attempt to ascertain the initial point of blockage in vivo by an agent such as methionine sulfoxide or cysteic acid. One would anticipate that cysteic acid might cause a pantothenic acid deficiency primarily and that this would not be the case with an agent such as hydroxyaspartic acid.

Methionine sulfoxide acts as growth inhibitor in certain bacterial systems by virtue of its capacity to inhibit the amidation of glutamic acid and transamination reactions in general; the first statement of inhibitory focus is based on the complete inactivity of methionine sulfoxide against gluta-

mine, the second by the failure of ketoglutaric acid to reverse the toxicity of this antimetabolite. Another metabolic system, namely the deamidation of glutamine, is inhibited by the alkylamides of glutamic acid, a conclusion based upon the fact that analogues such as glutamylhydrazine are not reversed by glutamine.

It is interesting that although aspartic acid does not reverse metabolite analogues of glutamic acid, for example, methionine sulfoxide, glutamic acid does reverse such aspartic acid displacers as cysteic acid and hydroxyaspartic acid. The implication is that glutamic acid formation from aspartic acid via transamination reactions must be prevented by the inhibitors or must be so slow as to be critical.

Two aspartic acid metabolite analogues have been studied in some detail, namely cysteic and hydroxyaspartic acids. The first of these, cysteic acid, functions in *Escherichia coli* systems by virtue of its capacity to prevent the decarboxylation of aspartic acid, a process which stops β -alanine formation and therefore pantothenic acid synthesis. Hydroxyaspartic acid possesses this capacity but also is probably a general inhibitor of transamination. This statement is based upon the failure of either β -alanine or pantothenic acid to completely reverse the inhibitory effect of hydroxyaspartic acid. Both β -alanine and pantothenic acid will completely reverse the action of cysteic acid. One confusing aspect of the study of cysteic acid inhibition is the marked capacity of glutamic contrasted to aspartic acid in the nullification of toxicity.

It is worthy of note that the action of cysteic acid is quite specific, because in general sulfonic acid analogues are nonspecific. There does not seem to be a pattern for effective metabolite analogues of either aspartic or glutamic acid. Two general classifications might be made: those with γ -carboxyl modification such as glutamylhydrazine and DL-N-(γ -glutamyl)-ethylamine and those with β -carbon change to hydroxyl or amino groups for example, *meso*-diaminosuccinic acid, hydroxyaspartic acid, and β -hydroxyglutamic acid. This is only a rough classification and it must be stated that no distinction can be made between the types from the standpoint of their biological action.

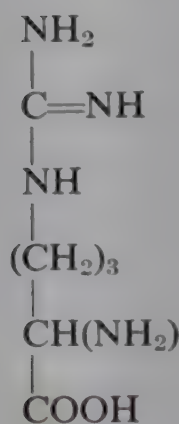
In conclusion, mention should be made of the effect of penicillin on the passage of glutamic acid across the cell wall. This transport, dependent upon a supply of energy, is inhibited by the antibiotic. It has been suggested that such blockage is the prime point of action of penicillin (Gale and Taylor, 1946, 1947). A similar mechanism might be associated with the antimetabolite action of any series of displacers.

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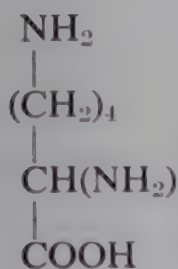
Chapter 9

ARGININE, LYSINE, AND HISTIDINE ANALOGUES



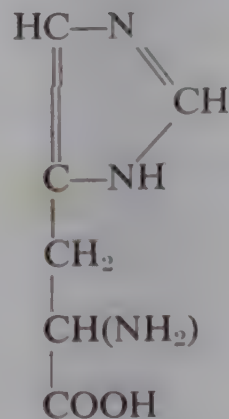
Arginine

(δ -guanidine- α -amino-
valeric acid)



Lysine

(α - ϵ -diaminocaproic
acid)



Histidine

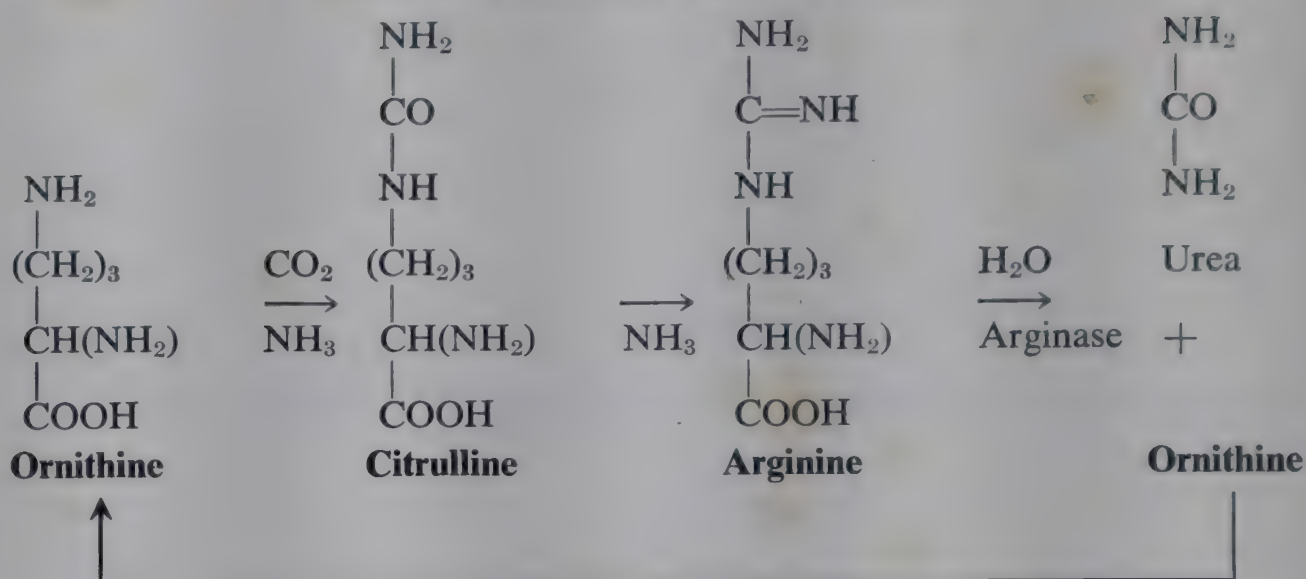
(β -4-imidazole- α -
aminopropionic
acid)

While the issue is debatable, there is evidence based upon the relative constancy of arginine, lysine, and histidine content of proteins which indicates that the basic amino acids form the nucleus of all proteins. If the hypothesis is correct, displacers for these amino acids might initially affect protein synthesis. In any event, it is certain that the protamines which characterize sperm cells and with nucleic acids form chromatin, are composed primarily of arginine, histidine, and lysine. While the other two basic amino acids vary, arginine is always present in protamines. Another point of interest in the group function of these amino acids lies in the fact that the ϵ -amino group of lysine is free in proteins and subject to deamination.

The three basic amino acids are listed as indispensable, with the proviso that histidine is not required for nitrogen balance in the adult human and arginine can be synthesized in the rat although not rapidly enough to meet demands for growth. Arginine is indispensable in the chick.

Arginine

Isotopic nitrogen is incorporated into arginine in the guanido group only, due to the metabolic activity of this moiety in urea formation. The following chart illustrates reactions involved in the Krebs urea cycle in the liver.



In this sequence, aspartic acid is the ammonia donor for the citrulline to arginine reaction which is catalyzed by ATP, cytochrome C, and Mg ions.

Arginine forms by anaerobic bacterial action ornithine and urea, and the former is decarboxylated to putrescine. There is a decarboxylase for arginine itself and its cofactor is pyridoxal phosphate.

The formation of guanidoacetic acid from arginine and glycine has been reported; subsequent methylation of this acid yields creatine and finally phosphocreatine which is the immediate energy source for muscular contraction.

Just as phosphocreatine plays a prime role in vertebrate muscle contraction, so phosphoarginine assumes this part in the invertebrate. Arginine is not acted upon by either D- or L-amino acid oxidase.

Considering the prime composition of the protamines in sperm, it is not surprising that a deficiency of this amino acid in the human being inhibits spermatogenesis even though a negative nitrogen balance was not observed. Therapeutically, lysine seems to be of little value in oligospermia. In the rat and other animals, arginine can be synthesized, with limitations. It is suggested that both the natural and unnatural forms of this amino acid may be utilized, but this has not been established.

The bacterial breakdown products of arginine include agmatine, ornithine, carbon dioxide, water, citrulline, succinic acid, and γ -butylene glycol. Several organisms are known to require arginine for optimal growth, namely *Clostridium sporogenes*, certain lactic acid bacteria, one mutant of *Escherichia coli*, and *Streptococcus zymogenes*. Either *Lactobacillus arabinosus* or *Lactobacillus casei* is used in the quantitative microbiological determination of this hexone base.

Lysine

In general, the amino groups of this amino acid are nonreactive as reflected in a failure of the incorporation of isotopic nitrogen. Lysine is in

fact the only amino acid which fails to incorporate either N¹⁵ or deuterium. It can give but cannot receive amino groups; its deamination is irreversible. It follows that the amino acid oxidases do not utilize lysine as substrate and cannot convert the D-form. Furthermore, α -aminocaproic acid cannot replace lysine in the diet of the vertebrate, an observation which establishes the fact that an amino group cannot be attached to the ϵ -carbon. The only known function of lysine is in protein formation.

Lysine is oxidized in the liver to α -aminoadipic acid which is in turn converted to α -ketoadipic acid and finally to glutaric acid. Deficiency of this factor causes growth defects, poor reproduction, and an anemia in animals. In humans, the deficiency state is characterized by negative nitrogen balance, weight loss, nausea, dizziness, and hypersensitivity to metallic sounds.

Bacterial decomposition of lysine results in the formation of ϵ -aminocaproic acid and cadaverine. Some lactic acid bacteria and *Streptococcus zymogenes* require lysine for optimal growth. *Leuconostoc mesenteroides* or a *Neurospora* mutant is used in the quantitative determination of this hexone base.

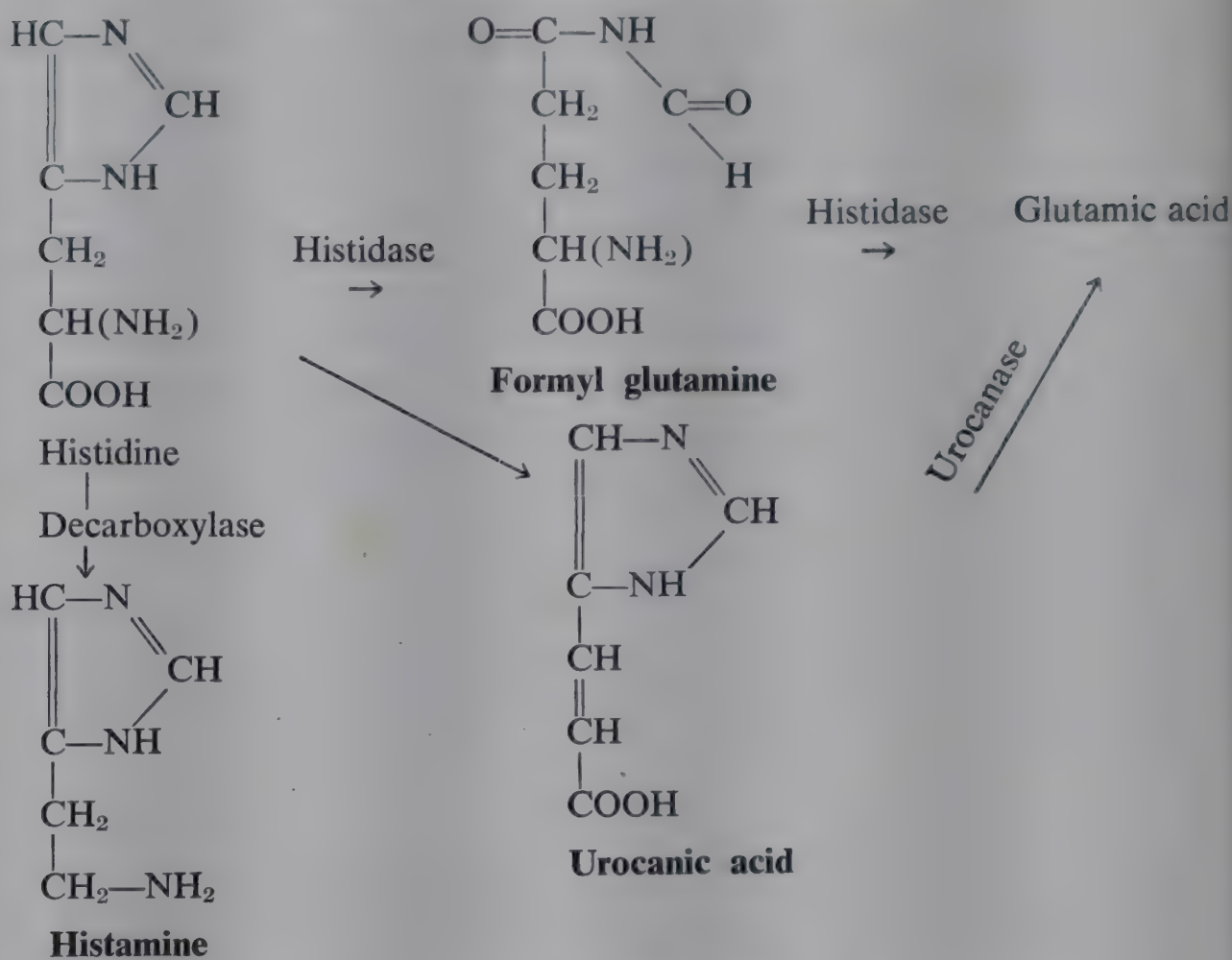
Histidine

DL- β -4-Imidazolelactic acid and imidazole pyruvic acid replace histidine in deficient diets, indicating that amination occurs. Such molecules as methylimidazole, hydroxymethylimidazole, imidazolecarboxylic acid, β -imidazolepropionic acid, and β -imidazoleacrylic acid cannot replace histidine. Isotopic nitrogen is incorporated into histidine, exclusively into the α -amino group. L-Amino acid oxidase will deaminate L-histidine, and while this amino acid accelerates the action of D-amino acid oxidase, its D-form will not serve as a substrate. This is not in keeping with numerous reports on the availability of the unnatural form.

Putrefactive anaerobic decomposition of histidine, which may occur in the gut, forms histamine, a powerful physiological depressor, implicated in shock, allergies, etc. The general path of metabolism of histidine in the animal is presented schematically on p. 176.

Two natural dipeptides of histidine are known. Carnosine, a combination of β -alanine and L-histidine, is found in muscle. It possesses a marked circulatory depressor action. Another such peptide is anserine, a compound of β -alanine and L-1-methylhistidine, which occurs in the muscles of geese. Still another physiological histidine derivative is ergothioneine, the betaine of thiolhistidine.

The breakdown products of histidine by bacteria include: β -imidazolelactic acid, β -imidazolepropionic acid, β -imidazoleacrylic acid, histamine, β -imidazole ethyl alcohol, CO₂ and NH₃. Some strains of *Corynebacterium diphtheriae*, some lactic acid bacteria and *Acetobacter suboxydans* require histidine for optimal growth.



Metabolite Analogues of the Hexone Bases

1. Metabolite Analogues of Arginine.
2. Metabolite Analogues of Lysine.
3. Metabolite Analogues of Histidine.
4. Recapitulation.

Metabolite Analogues of Arginine

The first studies of metabolite antagonism in this category were designed to ascertain the action of various amino acids and other compounds on arginase. Ornithine was first reported to inhibit the action of arginase (Gross, 1921). Subsequently cysteine was found to exert a similar action under certain conditions (Salazkin and Solovev, 1931, 1932). In 1936, Edlbacher and Zeller added glycine, alanine, and lysine to the list, the latter compounds being as powerful as ornithine. Then, Kitagawa (1939) found that canaline and several other amino acids were effective inhibitors, the most active compound being α -aminovaleric acid.

An extensive study of the inhibitory action of amino acids on arginase was presented in 1945 by Hunter and Downs. They reported the inhibition of arginase by all of the naturally occurring α -amino acids, but not by the unnatural forms, amino acids having amino groups in other than the α -position, urea or native proteins. An equation was developed expressing

the effect of varying concentrations of an inhibitor amino acid when the arginine concentration remained fixed. The equation is:

$$C = \frac{I - a}{1 - a}$$

I is the concentration of the inhibitor, a is the resultant "fractional activity," and C is the constant which is characteristic of each amino acid. By applying this equation to their data, they found that they were dealing with two types of inhibitors, the first being the monoamino acids, noncompetitive inhibitors, and the second the diamino acids (ornithine and lysine), competitive inhibitors.

L-Canavanine, an amino acid from jack beans, is subject to the action of arginase (Damodaran and Narayanan, 1950), is nontoxic to mice but toxic to dogs at levels of 200 to 400 mg. per kilogram of body weight (Appel, 1939) and is a potent inhibitor of L-arginine as tested on growth in certain wild strains of *Neurospora* (Horowitz and Srb, 1948). The potency of canavanine is reflected in the concentration necessary to stop growth in a

Table 1

METABOLITE ANALOGUES OF THE BASIC AMINO ACIDS

Compound	Activity	Test System	Comment	Reference
ARGININE				
Ornithine	+	Arginase	Activity	Gross, 1921; Hunter and Downs, 1945
Cysteine	+	"	Activity, probably nonspecific	Salazkin and Solovev, 1931
Natural α -amino acids	+	"	"	Edlbacher and Zeller, 1932; Hunter and Downs, 1945
Canaline	+	"	"	Kitagawa, 1939
α -Aminovaleric acid	+	"	"	"
Unnatural forms of α -amino acids	—	"	"	Hunter and Downs, 1945
Urea	—	"	"	"
β -Alanine	—	"	"	"
β -Amino-n-butyric acid	—	"	"	"
δ -Amino-n-valeric acid	—	"	"	"
ϵ -Amino-n-caproic acid	—	"	"	"
Canavanine	+	<i>Neurospora</i>	Growth	Horowitz and Srb, 1948

Table 1—(Continued)

Compound	Ac- tivity	Test System	Comment	Reference
LYSINE				
Arginine	+	<i>Neurospora</i>	Growth	Doermann, 1944
α -Amino- ϵ -hydroxy- caproic acid	+	Rats	Growth and anemia	Gingras et al., 1947 Page et al., 1947
Homolysine	$\pm$	<i>Streptococcus</i> <i>hemolyticus</i> <i>Staphylococcus</i> <i>aureus</i> <i>Escherichia coli</i>	Growth	Harris and Work, 1950
β -4-Piperidyl- alanine	$\pm$	"	"	"
2-(4'-Piperidyl)- 1-aminopropio- nyl-DL-leucine methyl ester	+	"	"	"
2-(4'-Piperidyl)- 1-aminopropio- nyl-p-amino- benzoic acid	+	"	"	"
α -Aminoadipic acid	—	Bacteria	Growth	Stevens and Ellman, 1950
L- α -Amino- ϵ - ureido-n-caproic acid	—	Rat Bacteria	Growth	"
DL-Piperidine-2- carboxylic acid	—	"	"	"
HISTIDINE				
Dopa	+	Histidine decarboxylase	Inhibition	Werle, 1942
β -2-Thienylalanine	+	"	"	Beiler et al., 1949
3-Benzothiophene- α -aminopropi- onic acid	+	"	"	"
Imidazoleamino- methyl sulfonic acid	+	"	"	"

sensitive *Neurospora*, namely 5.55×10^{-6} M. In general the specificity was high but in one strain L-lysine reversed the action. The canavanine-arginine antagonism is of the competitive type with a metabolite/inhibitor ratio of 3. Canavanine is therefore one of the most powerful metabolite antagonists known. Horowitz and Srb point out that the strains of *Neurospora* used were capable of synthesizing arginine and that a concentration of arginine

which provides good growth in an *arginineless* mutant did not counteract canavanine. They interpret this to indicate that canavanine does not interfere with arginine synthesis. While it is indicative, it is not positive proof of this point; inhibition of the synthesis of arginine might be superimposed upon an ability to prevent the metabolism or function of formed arginine.

Canavanine is not only a growth inhibitor for *Neurospora* but also for various strains of lactic acid bacteria and for *Escherichia coli* (Volcani and Snell, 1948). With these bacteria, the specificity was 100 per cent; lysine was ineffective. Furthermore, the inhibition was of the competitive type and occurred whether or not the organisms could synthesize arginine. With bacteria capable of utilizing citrulline and ornithine, these agents were able to reverse the action of canavanine. Neither canaline nor α -amino- δ -hydroxyvaleric acid inhibited utilization of ornithine as tested.

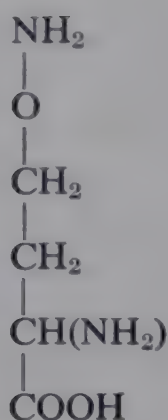
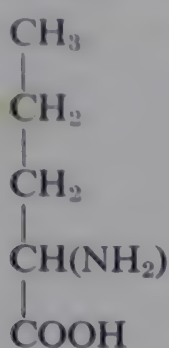
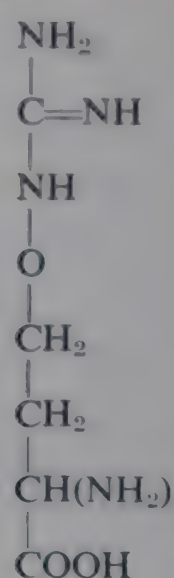
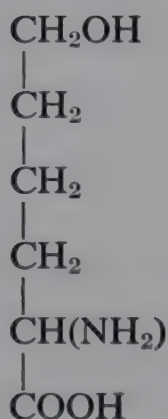
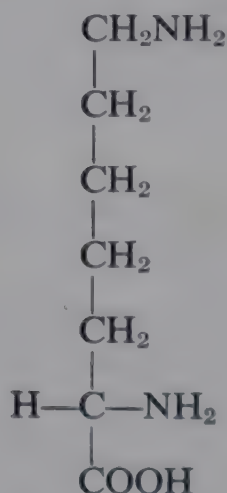
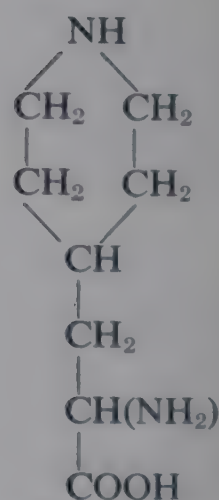
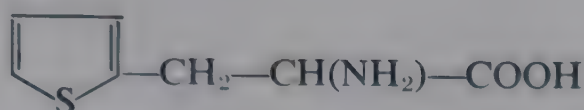
Potential applications of arginine metabolite analogues in chemotherapy are indicated by the capacity of arginine to antagonize penicillin when insensitive Gram-negative bacilli are used as test organisms (Shwartzman, 1946), and to counteract sulfonamides when insensitive streptococci are used (Gale, 1945).

Metabolite Analogues of Lysine

Using a mutant of *Neurospora crassa*, Doermann (1944) observed the specific competitive inhibition of arginine for lysine. This work is important because it represents a localization of the genetic development of susceptibility to arginine at a single gene of the first chromosome. It brings up the interesting potentiality of the possible action of metabolite analogues in inducing genetic modifications. Such phenomena have never been demonstrated but the possibility exists.

The second member of the series of lysine metabolite analogues to be reported was α -amino- ϵ -hydroxycaproic acid, which was found to exert a toxic effect in rats on lysine low diets as reflected in body weight (Gingras et al., 1947) and in the production of anemia (Page et al., 1947). It is probable that this analogue may be present in deaminized casein, which was shown by Hogan et al. (1941) to produce anemia in rats. Lysine did not reverse the toxic action of the caproic acid derivative, which leaves the question of displacement open. The action could be nonspecific or irreversible as studied.

Several series of molecules have been tested in systems which would reveal their inhibitory capacity and with one exception none of the molecules was very active (Stevens and Ellman, 1950; Harris and Work, 1950). The exception was a molecule tested by Harris and Work, 2-(4'-piperidyl)-1-aminopropionic acid, which when incorporated in a peptide linkage with p-aminobenzoic acid or DL-leucine showed a significant inhibitory capacity.

**Canaline** **α -Aminovaleric acid****Canavanine** **α -Amino- ϵ -hydroxy-
caproic acid****Homolysine** **β -4-Piperidyl-
alanine** **β -2-Thienylalanine****FIG. 1. Metabolite analogues of the basic amino acids.**

Metabolite Analogues of Histidine

Histidine which is the precursor of a very important pharmacological agent, namely histamine, has been given very little consideration as a model for potentially important metabolite analogues. Werle (1942) in his pioneering work on histidine decarboxylase observed the inhibitory effect of dihydroxyphenylalanine, epinephrine, and similar compounds. He ascribed this effect to the presence of phenolic hydroxyl groups in the various molecules tested. Certainly, phenolic compounds do inhibit histidine de-

carboxylase (Beiler et al., 1949) but this does not prove the absence of a more specific type of action. Beiler et al. (1949) tested several analogues of histidine and found a number effective (see Table I). Of these β -2-thienylalanine most closely resembles histidine in structure and it was the most active. It is seen that this promising field has been quite neglected.

Recapitulation

It is only necessary to reflect on the possible molecular structures which might well possess antiarginine activity to realize how limited have been studies in this field to date. Investigations of canavanine have disclosed that this molecule acts as a substrate for arginase, is of low toxicity and yet functions as a powerful inhibitor of *Neurospora* and bacteria. In vivo studies of the antibacterial potency of this molecule have not been made but it is clear that it typifies a compound which is ideally suited to chemotherapy. The activity of α -aminovaleric acid as an inhibitor of arginine indicates a tool which could probably be used to ascertain in vivo the effect of blockage of the Krebs urea cycle. Another potentiality would be the study of the effect of metabolite analogues of arginine on the reproductive capacities of vertebrates. It is known that deficiency of this factor inhibits spermatogenesis.

The competitive antagonism between arginine and lysine, a common phenomenon between amino acids, suggests as a theory that the toxicity of high levels of any amino acid may not be due to an action of the toxic amino acid *per se* but rather to its displacement of some structurally related amino acid from a vital enzyme site. Extension of work along these lines should prove fruitful. α -Amino- ϵ -hydroxycaproic acid is doubtless a direct metabolite antagonist of lysine but the point has not been established. The metabolic inertia of this basic amino acid should make it a profitable target for displacers but if it is true that its sole function is protein formation, such studies would prove relatively unrewarding. The symptom of hypersensitivity to metallic sounds manifested in lysine deficient patients might well form a clue through the use of displacers to the study of the role played by this hexone base in nerve physiology.

While the inhibitory action of dihydroxyphenylalanine on histidine decarboxylase has been attributed to the phenolic hydroxyls present, it seems that direct structural similarity to histidine may well play a role in view of the capacity of β -2-thienylalanine to function as a displacer. The pharmacological significance of histamine dictates the necessity of extensive studies of inhibitors of this decarboxylase. It seems logical to assume that effective agents would prevent the development of shock and perennial allergies by stopping histamine formation. Such drugs might very well extend the therapeutic armamentarium currently available. This is a desirable goal in view of the side actions manifested by all of the so-called antihistamines. Specu-

lation on molecular modifications which might lead to the creation of effective metabolite antagonists of histidine is not in order but it is certain that dozens of displacers await discovery.

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Chapter 10

PROTEINS AND PROTEIN ANTAGONISMS

1. Protein Structure.
2. Immunological Phenomena.
3. Interference Phenomena Among Viruses.
4. Protein Enzyme Inhibitors.
5. Antagonisms of Protein or Polypeptide Hormones.
6. Comment.

Protein Structure

Any review of proteins must logically include polypeptides, particularly those of a more complex nature, as these units are formed by the condensation of amino acids or by the hydrolysis of proteins and may be regarded as intermediates in the integration and disintegration of protein macromolecules. The structural similarity of polypeptides with intact protein indicates the potential function of many of these units as biological antagonists of their more complex relatives. In the American classification of proteins, polypeptides are regarded as derived proteins.

In this classification, in addition to the derived proteins there are two other classes: the simple proteins and the conjugated ones. The simple proteins are those which on hydrolysis yield only α -amino acids; the conjugated contain in addition to the amino acids a nonprotein unit, i.e., the nucleic acid moiety of the nucleoproteins, the carbohydrate radical of the glycoproteins, the phosphoric acid of the phosphoproteins, etc.

The potentialities for displacement phenomena are legion with molecules of such complexity. This is particularly true of the conjugated proteins wherein the nonprotein moiety might by modification easily result in the creation of an antagonist. Knowledge of protein structure is limited but one fact dominates the entire pattern and it is the peptide linkage. To be sure, the peptide linkage alone is not adequate but it is the foundation upon which other concepts have been erected.

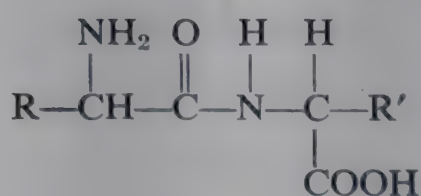
Biological antagonism is so intimately associated with protein units that an insight into modern thought on protein structure is bound to prove of value in the consideration of the general phenomenon of antimetabolites.

Kossel (1896) promulgated a protamine nucleus hypothesis which suggests that the nucleus of all proteins is composed of basic amino acids. Ap-

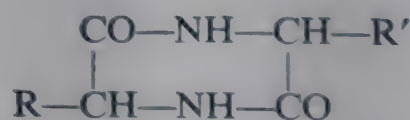
proximately 20 years later, both Abderhalden and Bergmann introduced alternate theories. Abderhalden (1923, 1924) thought of proteins as constituted of cyclic substituted diketopiperazine rings held together by secondary valence forces. His position was challenged by Levene and Bass (1928, 1929) who through studies of racemization speeds concluded that the odds were against the existence of any diketopiperazine units in casein. The concept of Bergmann (1924, 1925) is essentially similar to that of Abderhalden except in its emphasis on the hydroxyamino acids and their effect on the protein structure. He proposes the "iso" and the "allo" forms of the diketopiperazine rings.

Again, Troensegaard (1944) offers a challenging concept based upon the supposed existence within the protein of pyrrole rings. Other ring systems within the structure of proteins have been hypothetically asserted; i.e., dihydropyrazine rings (Karrer and Gränacher, 1924). Finally, the Wrinch (1936, 1937) proposal of a "cyclol" pattern with diazine and triazine hexagons built into sheets of amino acid aggregates, monomolecular in depth and polymolecular in breadth, held together by intermolecular attraction has been well received.

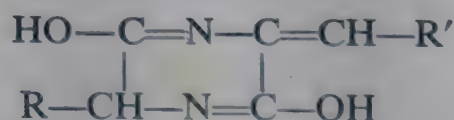
While no general theory of protein structure stands accepted, the structural classifications of these macromolecules into the categories of fibrous and globular is acknowledged. Meyer and Mark (1928) from studies of



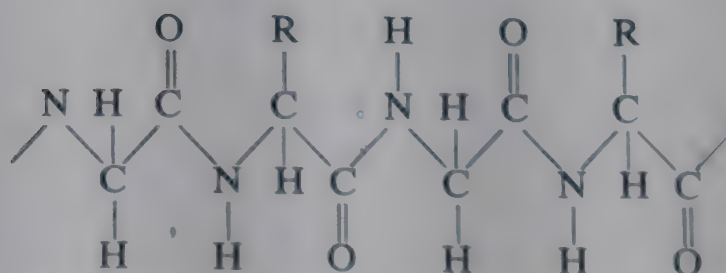
Peptide



Diketopiperazine



"Iso" form of a diketopiperazine



Polypeptide chain of a fibrous protein

FIG. 1. Protein structure.

x-ray patterns found repetitions spaced at 7 Å. units, which as indicated by Gortner and Gortner (1949) is roughly two times the skeletal axis of 3.67 Å. units reported by Corey (1940) for a single amino acid. These facts caused Meyer and Mark to offer as the structure of the fibrous protein, silk fibroin, the polypeptide chain shown in Fig. 1. Under certain conditions, fibrous proteins of the keratin type can be converted from the α -keratin to the β -keratin form (Astbury and Street, 1931). In the course of this transfer the protein is stretched to three times its original length. It is apparent that the folded main chains of the molecule are straightened to a degree and that the surface characteristics would be materially altered. It is probable that proteins existing in fibrous and globular forms, as they do in muscle, may well respond differently to biological antagonists. The surface structural characteristics offering reaction sites in one form will not necessarily present these same sites to a fixed structural unit when it is transformed. Thus, reaction sites may vary with the functional state of the protein involved whether it be an enzyme, muscle fiber unit, or any other biologically significant protein unit.

Vital factors in surface characteristics would be the R substituents in the general amino acid formulation, $R-CH(NH_2)-COOH$. Furthermore, the $-NH-$ and the $-CO-$ groupings would play a vital role, particularly if these units actually form the basis of the forces of attraction between protein layers through their electronegative and electropositive nature.

Little is known of the structural characteristics of the globular proteins but it can be assumed that the peptide chains must be bent, folded, turned, and twisted so that the chains lie in parallel axes in three dimensions.

Another point of tremendous importance to the concept of biological antagonism is the theory of recurring amino acid units as proposed by Bergmann (1938). He states "Every individual amino acid residue in the peptide chain of the protein molecule recurs at constant intervals . . . in a characteristic and periodic manner throughout the entire polypeptide chain." Different proteins have varying individual amino acid periodicity. It is not improbable that the distinctive protein feature of recurring amino acid patterns will form the basis of a science of antagonism. It will be the surface characteristics of the protein macromolecule which will determine displacement of cofactors, substrates, and of every phenomenon of antagonism. Correlation studies of protein surface structure and antimetabolites should prove most rewarding.

Furthermore, the magnitude of potential surface structures which should roughly correspond to the number of possible proteins gives some idea of the infancy of the science of biological antagonism. Gortner and Gortner (1949) indicate that if it is assumed that proteins are made up of only

20 amino acids, each amino acid present only once in a single chain, the possible number of permutations would be 24×10^{17} .

Immunological Phenomena

The science of immunology is a subdivision of the science of biological antagonism. Sevag (1945) in his treatise "Immuno-Catalysis" has presented the subject from that standpoint. It is his contention that antigenic proteins are enzymatic in nature and that the product of their activity is the specific antibody. The function of the specific antibody is to neutralize the activity of the antigen.

To Sevag, enzymes and antigens are one and the same, a group of biocatalysts. The substrate for the action of the antigen as an immuno-catalyst is serum globulin and the antibody is a modified form thereof.

There is a parallel between the appearance of antibodies and the increase in serum globulin but opinions differ as to the nature of this increase. Liu et al. (1937) contended that the increase was partially due to antibody but Bjørneboe (1939) felt that the entire increase was a reflection of antibody protein. Sevag (1945) holds that while there is a general parallel between antibody production and serum globulin levels, at least a portion of the increase is in a nonspecific protein.

The antibodies are γ -globulins by every applicable criterium including molecular weight, sedimentation rate, electrophoretic mobility, isoelectric points, etc. Normal and antibody globulins have been analyzed and there is no detectable difference in amino acid composition or total nitrogen (Marrack, 1938).

In spite of the apparent chemical identity of normal and antibody globulins, they are serologically distinct. It is proposed to establish the configurational variation responsible for the immunological characteristics as reflected in various phenomena. One point of importance is the possible complete synthesis of antibodies from amino acids. The dynamic state of proteins does however offer the potentiality that configurational modifications might be introduced under the proper stimulus without resort to complete *de novo* synthesis.

The method of formation of the antibody is of great importance to an understanding of biological antagonism, as it represents an instance in which a molecular unit catalytically produces a substance which is the biological antagonist of the catalyst. Pauling (1940) has offered a concept based upon the folding of the polypeptide chain of the antibody precursor in the presence of the antigenic molecule. The formed antibody will then possess a configuration complementary to the antigen. The biological forces at work would be the ordinary short-range forces known to exist between simpler molecules.

It is indicated by Pauling that certain proteins, such as trypsin and

hemoglobin, lose their specific properties under the action of denaturing agents, and subsequently regain their biological activity. He interprets this as an indication that the molecule recoils to its normal, stable configuration.

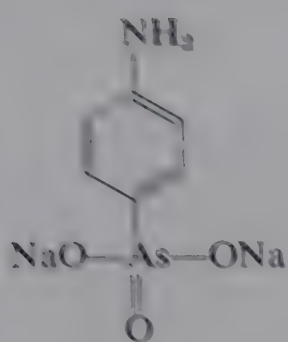
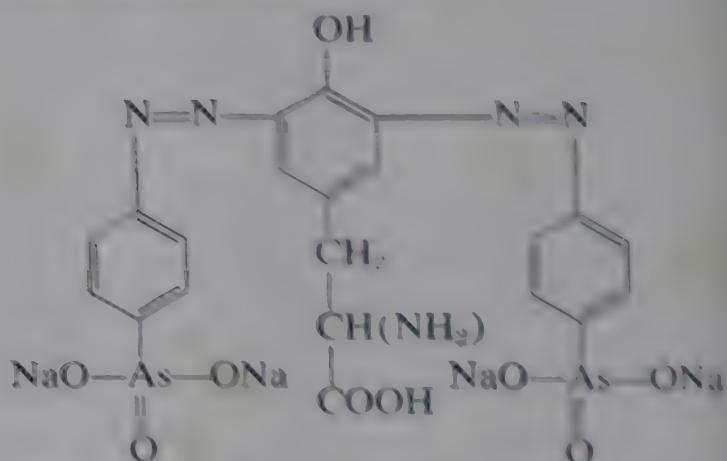
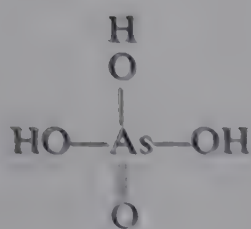
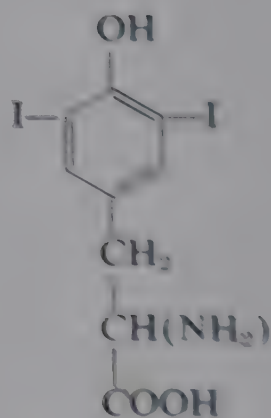
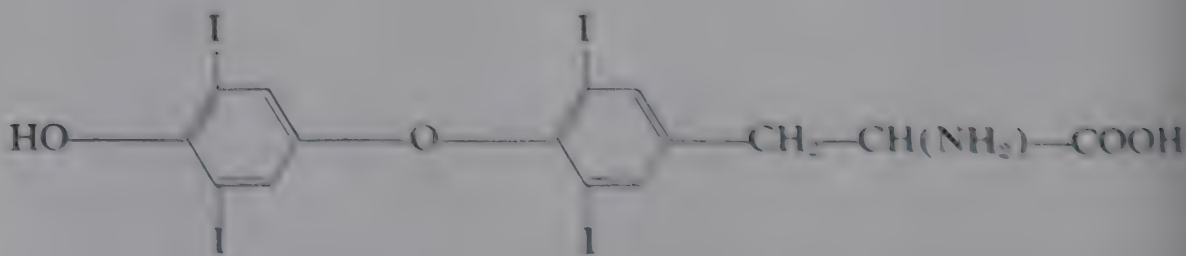
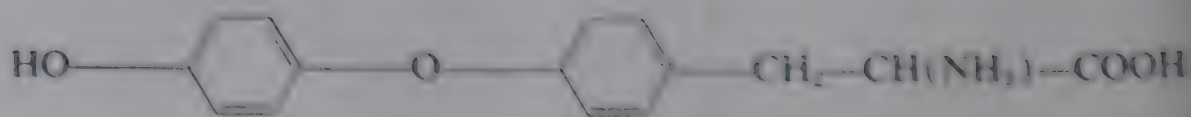
The substrate for the action of the antigen is the γ -globulin which is assumed to possess a polypeptide chain capable of folding in a number of alternate ways. When the polypeptide is synthesized in the cell, in the absence of the antigen, it will coil to form the normal γ -globulin. In the presence of the antigen, the conditions within the cells are such as to cause the folding of the chain into a form stable by virtue of the presence of the antigen.

The basis for the contention that short-range forces were primarily responsible for the building of antibody molecules was based upon the work of Landsteiner (1947), Haurowitz (1942), Pressman et al. (1948), and others using antigens prepared by conjugating chemically a hapten with a protein molecule. Using these artificial antigens, antisera could be prepared and cross-reactions studied. Moreover, the capacity of the low molecular weight hapten to interfere with the precipitation of the antibody and the hapten conjugated antigen, discovered by Landsteiner, was interpreted by Pressman et al. (1948) as due to competition between hapten and hapten conjugated antigen for the complementary sites on the antibody. This is another manifestation of biological antagonism.

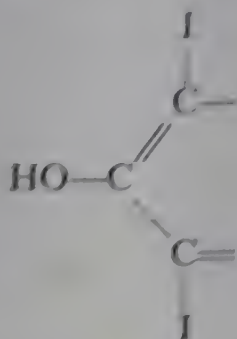
Landsteiner (1920) observed that the formation of a precipitate by atoxyl-azo-protein and antiserum was prevented by the addition of an excess of relatively simple substances containing atoxyl, i.e., diazotized atoxyl coupled to tyrosine, by atoxyl itself and even by arsenic acid.

In 1924, Landsteiner reported the ability of these simple hapten units to prevent the development of anaphylactic shock in guinea pigs sensitized with atoxyl-azo-protein. The displacement capacity of the atoxyl was operative either *in vitro* or *in vivo*.

In support of the theory of biological relativity, no absolute specificity should exist in the serology phenomena under consideration. The work of Wormall (1930) and others establishes this point. The antigenic agents used in his work were made by the introduction of halogens into proteins mainly in the benzene ring of tyrosine, ortho- to the p-hydroxy grouping. The iodo- and bromo-proteins produced in this manner gave strong cross-reactions which are manifestations of only relative specificity. This situation also held with the simple hapten interferences with precipitate formation when these antigens were mixed with the corresponding antisera. Diiodo-tyrosine, dibromotyrosine, and dichlorotyrosine inhibited with potency decreasing in the order listed. As an indication of the relative specificity, potassium iodide, o-iodophenol, tyrosine, and other amino acids were without effect.

**Atoxyl****Diazotization product of atoxyl and tyrosine****Arsenic acid****Diiodotyrosine****Thyroxine****Thyronine****FIG. 2.** Simple hapten inhibitors of antigen-antibody reactions.

Later, Snapper and Grunbaum (1936) extended these observations and reported that all aromatic compounds containing the grouping



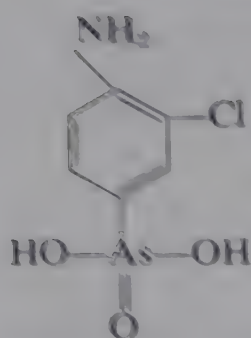
were inhibitory. Thyroxine was inhibitory, thyronine was not.

This interference phenomenon involving simple haptens is one of the most extensively studied of all displacement reactions. Furthermore, it is so closely related to the basic protein structures which underlie most biological forces and antagonisms that it should logically provide understanding of phenomena not yet elucidated in regard to other aspects of metabolite displacements and biological antagonism.

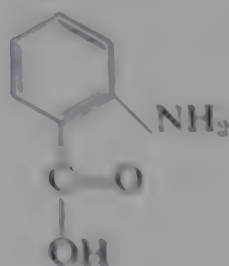
Another example of this inhibition by simple haptens was provided by Landsteiner and van der Scheer (1931) who found that the reaction between p-aminobenzenearsonic acid antibody and the homologous antigen was blocked by p-aminobenzenearsonic acid, o-aminobenzenearsonic acid, and 3-chloro-4-aminobenzenearsonic acid. In this same paper, they described the blockage of precipitate formation between o-aminobenzoic acid antibody and the homologous antigen by benzoic acid, by benzoic acid substituted either in the o- or m-position with $-\text{CH}_3$, $-\text{Cl}$, $-\text{Br}$, or $-\text{NO}_2$; by thiophenecarboxylic acid; naphthoic acid; and cyclohexanecarboxylic acid. Some interference was even obtained with fatty acids, specifically caproic and heptylic acids.

In the case of the pyrazolone compounds Erlenmeyer and Berger (1934) found that the position of the double bond affected specificity. The antigen formed by diazotized 1-phenyl-2,3-dimethyl-4-amino-5-pyrazolone would not react with its antiserum in the presence of other compounds of the pyrazolone type with the double bond between the 3- and 4-carbons. Such molecules with the double bond between the number 2- and 3-carbons did not prevent the reaction.

It is probable that in those instances of compounds manifesting antagonism in other biological systems, the preparation where possible of diazotized protein couples would show that the molecules antagonistic in enzymatic reactions would be so in simple hapten interference phenomena. It is unfortunate that to date no experiments have been reported in which antigens were created using β -2-thienylalanine and an attempt was made to block the precipitation reaction by the use of phenylalanine, tyrosine, and



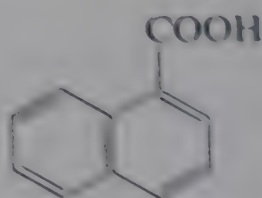
3-Chloro-4-amino-
benzenearsonic acid



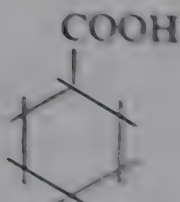
o-Aminobenzoic acid



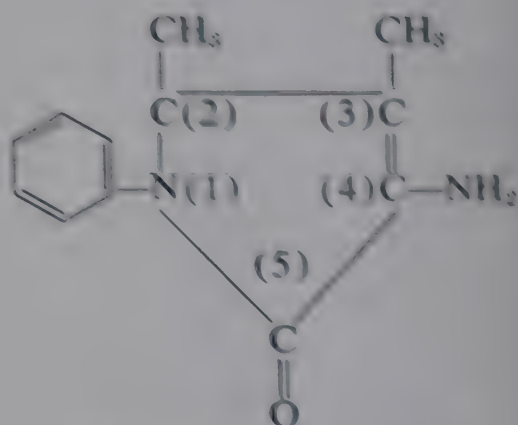
Thiophenecarboxylic acid



α -Naphthoic acid



Cyclohexanecarboxylic acid



1-Phenyl-2,3-dimethyl-4-
amino-5-pyrazolone

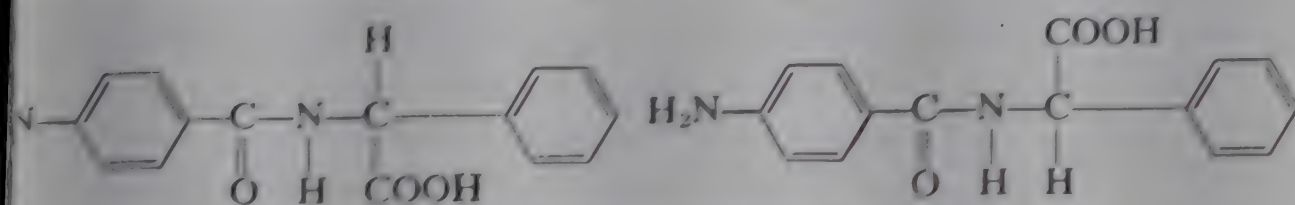
FIG. 3. Simple hapten inhibitors of antigen-antibody reactions.

similar molecules. This is only a single example of an approach to the problem of biological antagonism which could be extended to studies with all systems wherein one member could be coupled through diazotization with a protein.

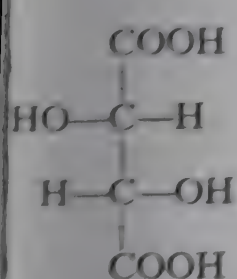
The importance of this hapten interference mechanism makes pertinent a review of other examples. One of these, important in the study of protein specificity, was made with peptides as hapten units (Landsteiner and van der Scheer, 1932). The reaction of antigen prepared with p-aminobenzoyl-glycyl-leucine and its antibody was inhibited by p-aminobenzoyl-glycyl-leucine and p-aminobenzoyl-leucyl-leucine, but not to any degree by the corresponding glycyl-glycine or leucyl-glycine compounds. Landsteiner and van der Scheer (1934) from studies on similar systems with peptides con-

aining up to five units concluded that the predominant effect is that of the terminal amino acid. The nature, order, and number of the other non-terminal amino acids is of comparatively little importance.

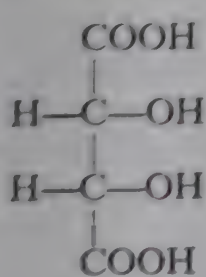
The effect of spatial configuration upon the hapten blockage phenomena was determined by Landsteiner and van der Scheer (1928) in their experiments with phenyl-(p-aminobenzoylamino)-acetic acids. Some overlapping of the hapten blockage phenomena occurred. Later, these investigators (Landsteiner and van der Scheer, 1929) used the optical isomers of tartaric acid in similar researches by forming the tartranilic acids and coupling these



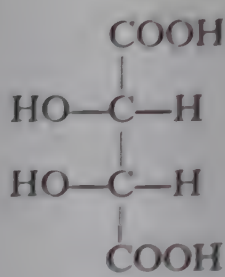
Phenyl-(p-aminobenzoylamino)-acetic acids



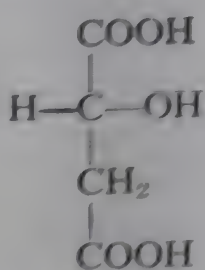
L-Tartaric acid



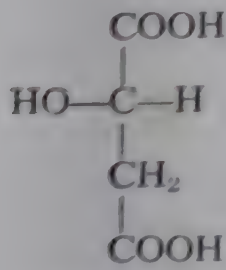
Meso-tartaric acid



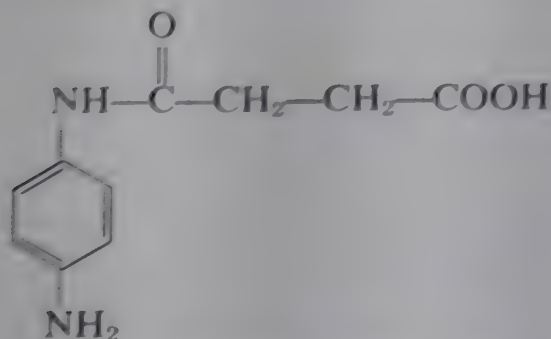
D-Tartaric acid



D-Malic acid



L-Malic acid



p-Aminosuccinanilic acid

FIG. 4. Simple hapten inhibitors of antigen-antibody reactions.

units with protein. The meso form gave evidence of greater specificity than the D- or L-isomers with which overlap was significant. The optical isomers of malic acid were active in the system under study, the specificity being relative and directed toward the corresponding tartaric acid molecule.

Furthermore, the *cis* but not the *trans*-compounds of derivatives of maleic, citraconic, fumaric, and mesaconic acids exhibit the inhibition reaction with the antigen and antisera prepared from p-aminosuccinanilic acid (Landsteiner and van der Scheer, 1934a).

These studies correlate rather well with direct displacement work. With succinic dehydrogenase as the test system, malonic acid displaces succinic. The malonate inhibition can be reversed by fumaric acid in the pyruvate oxidation system. Under other conditions, malic, fumaric, and oxalacetic acids can act as antagonists of succinic acid (see Chapter 1). This tends to substantiate the position that simple hapten blockage studies may well represent an excellent tool for the development of new metabolite antagonists.

Another type of simple hapten study which can be correlated to some degree with direct enzymatic displacement studies centers around the monosaccharides. p-Aminophenol- β -glucoside and p-aminophenol- β -galactoside were coupled with proteins and used as antigens (Avery and Goebel, 1929). The precipitation reaction was inhibited by the corresponding homologous hexose but not by the heterologous hexose. The work of Woolf, Marrack, and Downie (1936) again shows the displacement specificity of carbohydrates and their derivatives. They found that euxanthic acid (a glucuronic acid compound) attached to a protein precipitates with Type II antipneumococcal serum. This reaction is blocked by glucuronic acid but not by galacturonic nor mannuronic acid. The α - and β -glucosides used in similar studies (Avery et al., 1932) did not manifest the same degree of specificity. The precipitate formation by an antibody and a homologous antigen was blocked by the homologous glucoside but the cross-reaction was blocked by both the homologous and the heterologous glucoside.

A parallel phenomenon has been recorded (Kuhn, 1923) which indicates the inhibition of β -h-fructosidase by fructose and β -glucose but not by α -glucose; and the blockage of taka saccharase by α -glucose and not by β -glucose nor fructose. Chapter 1 reviews similar observations in this class. Once again, the correlation between displacement phenomena as seen with enzymes and the simple hapten precipitation inhibition reaction is clear.

Goebel et al. (1934) have used a series of disaccharides in these simple hapten inhibition studies. Maltose, cellobiose, and gentiobiose were used. The results indicated that the terminal hexose had the dominant effect, although the stereochemistry of the bond between the hexoses was important. In the same year this group (Goebel et al., 1934a) reported that modification of the carbohydrate in such antigens altered specificity reactions.

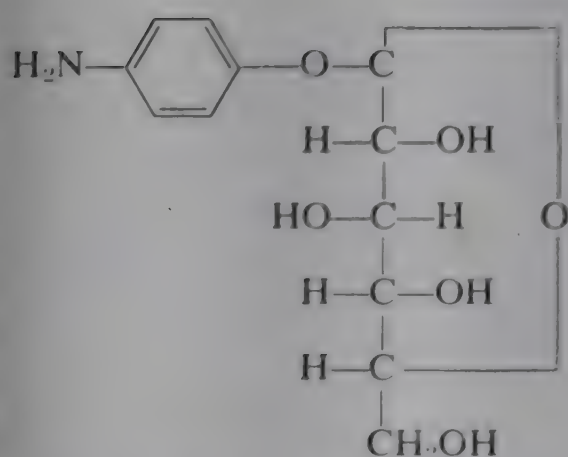
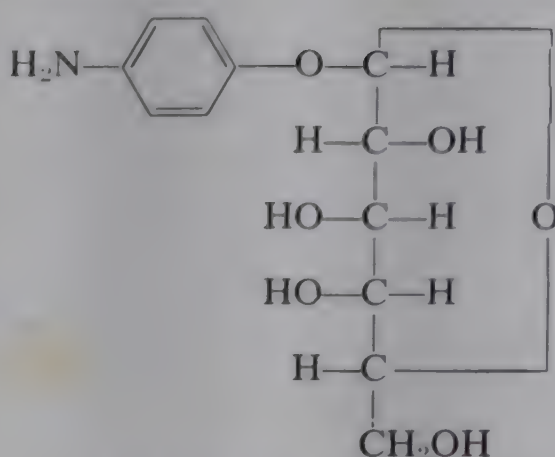
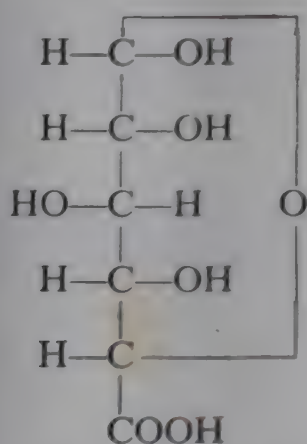
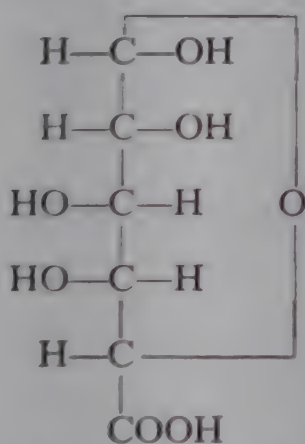
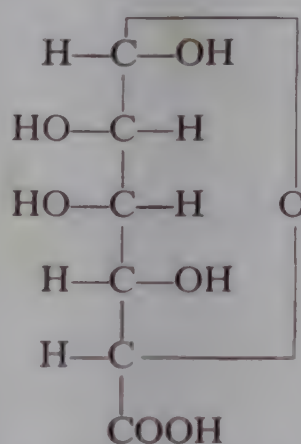
**p-Aminophenol- β -glucoside****p-Aminophenol- β -galactoside****Glucuronic acid****Galacturonic acid****Mannuronic acid**

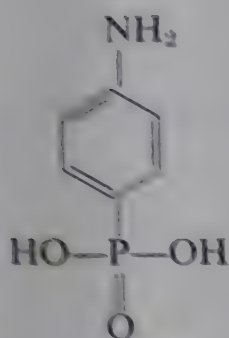
FIG. 5. Simple hapten inhibitors of antigen-antibody reactions.

Inasmuch as there is a direct correlation between enzymatic inhibition by structurally similar molecules and the simple hapten blockage phenomena, it is of exceptional interest to note that the same factors seem operative in the specific selection of molecules for incorporation into crystals. Erlenmeyer and Berger (1932) initially made record of this fact. The incorporation of molecules into crystals is a more specific phenomenon than that of immunological cross-reactions and interference. There are examples of antigen cross-reactions between molecular species which cannot replace each other in crystals.

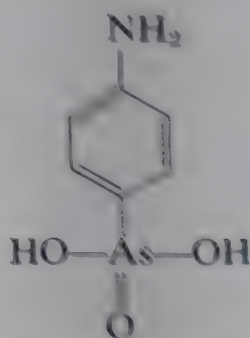
As an example of the Erlenmeyer and Berger concept, the molecules given in the top row of Fig. 6 form mixed crystals and those given in the second row are immunologically virtually equivalent in the simple hapten interference system (Landsteiner and van der Scheer, 1927). Similarly, by physical criteria and by immunological reactions, benzene and thiophene are identical while furan is more remote in its resemblance (Erlenmeyer and Leo, 1933; Landsteiner and van der Scheer, 1931; Berger and Erlenmeyer, 1933).



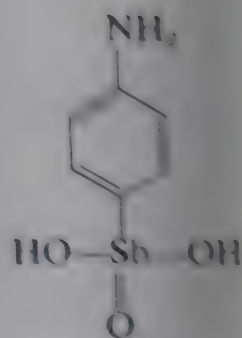
Equivalents as haptens



Phosphanilic acid



Arsanilic acid



p-Aminophenyl-
antimonic acid



Mixed crystal formers

FIG. 6. Mixed crystal formation and hapten inhibitors.

The findings with the benzene, thiophene, and furan rings are of interest in view of the studies on replacement of these rings in modifications of metabolites and the resultant displacement power. As an example, β -2-thienylalanine and β -2-furylalanine are effective antagonists of phenylalanine in bacterial systems (duVigneaud et al., 1945; Herz et al., 1947). It is not improbable that molecules forming mixed crystals will make displacing agents for each other in biological systems. This would certainly be a simple and rather neat correlation of a purely physical phenomenon with one of biological nature.

While there are a limited number of instances currently known in which mixed crystal formers, simple hapten blockade molecules, and enzymatic inhibitors perform in each sphere, there are additional examples of the occurrence of the first two as related interaction systems. The isomorphism of the salts of phosphoric and arsenic acids and the dissimilarity of anti-

sulfonic acid are reflected in the cross-reaction and inhibition phenomena seen when p-aminophenylarsenic (arsanilic acid), p-aminophenylphosphoric (phosphanilic acid), and p-aminophenylantimonic acids are used as determinant groups (Erlenmeyer and Berger, 1932a). Again, sulfuric and selenic acids are isomorphous and the reaction of p-aminophenylsulfonic acid antigen with its antibody is blocked by both benzene sulfonic acid and benzene selenic acid (Erlenmeyer and Berger, 1933). In this latter instance, there is a rough correlation with the observations of Green and Bialchowsky (1942) who found that in general the substitution of sulfur by selenium or tellurium results in compounds whose antibacterial properties are related to those of the corresponding sulfur molecules.

Beyond question, the studies carried out with the simple hapten inhibition phenomena and cross-reactions in immunological systems offer detailed information applicable to the entire field of biological antagonism.

Lecithinases

Just as there are antigens functioning enzymatically (Sevag, 1945) so there are enzymes which are known toxins. Lecithinase A is a constituent of venoms with the capacity for the conversion of lecithin to lysolecithin, the lytic agent for red cells. Lecithinase B acts on lysolecithin to form stearic acid and glycerophosphoric ester. The products of the action of this enzyme are not toxic in any manner. Here then is an example of one enzyme antagonizing the toxic action of another by utilizing as substrate the toxic product of the action of the first enzyme.

The alpha toxin of *Clostridium welchii* has been found to be an enzyme which hydrolyzes lecithin (Macfarlane and Knight, 1941); a lecithinase. There is in general a correlation between the production of this enzyme and the virulence of the organism (Evans, 1945; Kass et al., 1945). Evans (1945) has indicated this lecithinase is mainly responsible for the toxic manifestations of gas gangrene.

The investigations which followed the identification of toxin and enzyme have brought pathology into the realm of enzymology, and immunology clearly into the sphere of biological antagonism. Zamecnik et al. (1945) interpreted the effects of a bacterial toxin on tissues in terms of its enzymatic activity. Later, this same group, having developed a manometric method for measuring the activity of the *Clostridium welchii* lecithinase (Zamecnik et al., 1947), determined the antagonistic power of phosphatidyl serine and phosphatidyl ethanolamine (Fig. 7) against the enzymatic hydrolysis of lecithin. A fraction of the inhibitory effect of phosphatidyl serine was attributed by these workers to calcium chelation. Davis and Dubos (1946) had reported the blocking power of ox brain cerebroside, sphingomyelin, and a sorbitol-mannitol-oleic acid ester for this lecithinase.

The next step in the development of this demonstration of the enzymatic

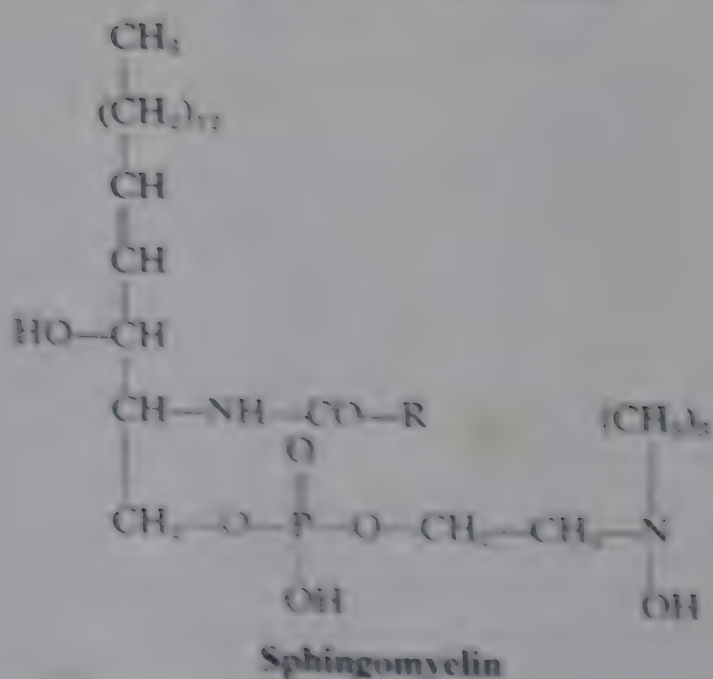
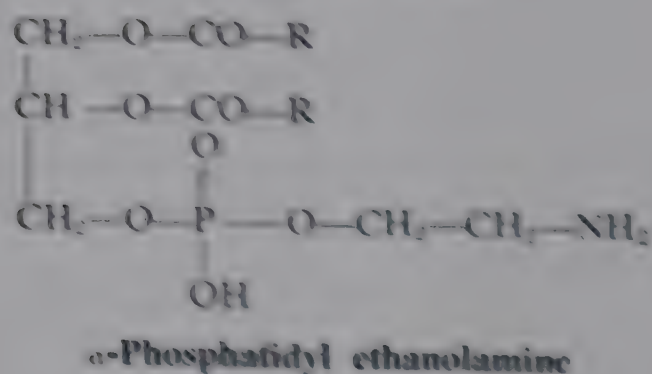
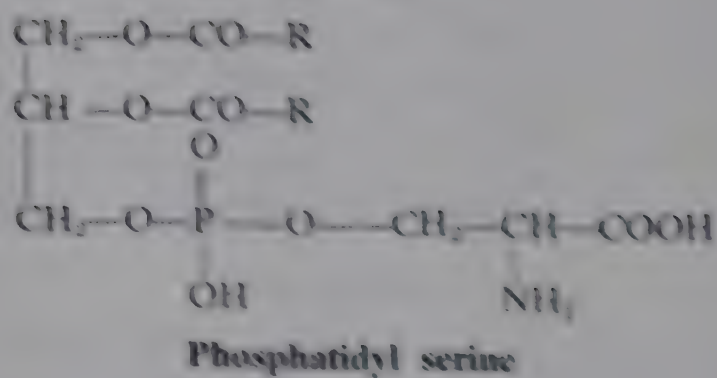
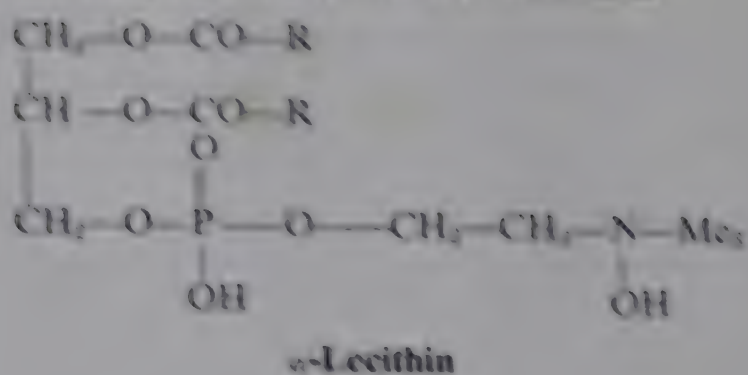


FIG. 7. Inhibitors and substrate for *Chlamydomonas* acyl-CoA lecithinase.

ture of toxins was the establishment by Macfarlane and Knight (1941) of the capacity of antibody to block the enzymatic activity of the toxin. From this, Zamecnik and Lipmann (1947) concluded that the antibody and lecithin must react with analogous regions of the lecithinase-toxin molecule and that competitive phenomena should be demonstrable. They were able to prove that lecithin prevents antitoxin from combining with toxin-lecithinase. In their experiments, if they first exposed the lecithinase to its substrate, lecithin, and then added the antitoxin, the enzymatic reaction continued but was slowly decelerated. By contrast, if the toxin-lecithinase was first brought into contact with the antitoxin, the reaction was completely blocked. Finally, if the lecithinase were exposed to both lecithin and antitoxin simultaneously the enzymatic process proceeded at a fractional rate but decreased with time elapsed. In relation to clinical observations, Zamecnik and Lipmann point out the ineffectiveness of the late administration of *Clostridium welchii* antitoxin, which would correlate well with the probable prior combination of the lecithinase with its normal substrate and the difficulty of displacement of the substrate by the antitoxin.

The fact that the lecithin and antitoxin relationship exists with the lecithinase of rattlesnake venom has been demonstrated (Zamecnik and Lipmann, 1947) and found to differ quantitatively but not qualitatively from that for *Clostridium welchii* lecithinase. Other lecithinases have been discovered in *Clostridium oedemanticus* (Macfarlane, 1948) and in *Clostridium ordellii*. It would appear therefore that the substrate and antibody system is general in nature but even if restricted to the lecithinases would be applicable to many pathogenic bacteria.

Hemagglutinin

Hemophilus pertussis is capable of agglutinating erythrocytes of various species (Keogh, North, and Warburton, 1947) and attempts at elucidation of the basic mechanism have revealed much of fundamental interest to the general study of biological antagonism. The hemagglutination by *Hemophilus pertussis* resembles that caused by viruses of the influenza group, indicating a similarity between the viral and bacterial immunological phenomena in a limited sense.

In 1942, Fisher discovered the ability of ether and acetone extracts of human red cells to inhibit the hemagglutinating action of *Hemophilus pertussis* supernatants, and the following year he identified the active principle as cholesterol. It is probable therefore that the receptor site for the hemagglutinin molecule is the cholesterol contained in the red cell surface. In his experiments, Fisher found cholesterol, ergosterol, calciferol, and corn germ sterol (assumed to be stigmasterol) active inhibitors, while androsterone, cholic acid, desoxycholic acid, 7-ketocholesterol acetate, and cholesterol palmitate and oleate were inactive. The active sterols differed from the in-

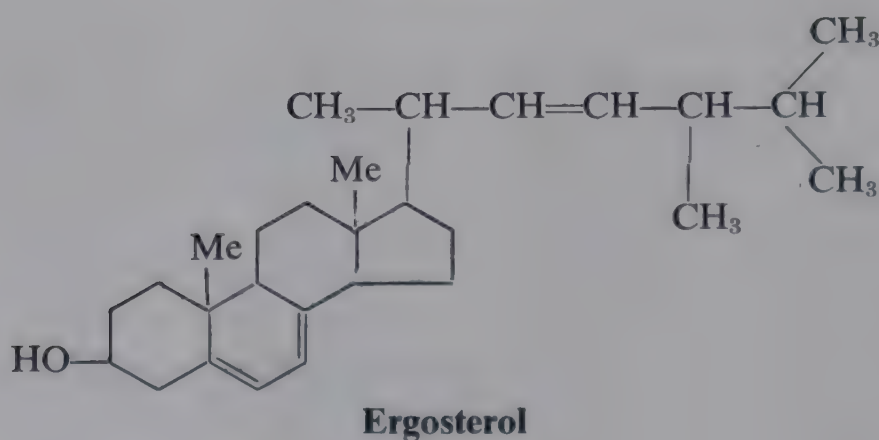
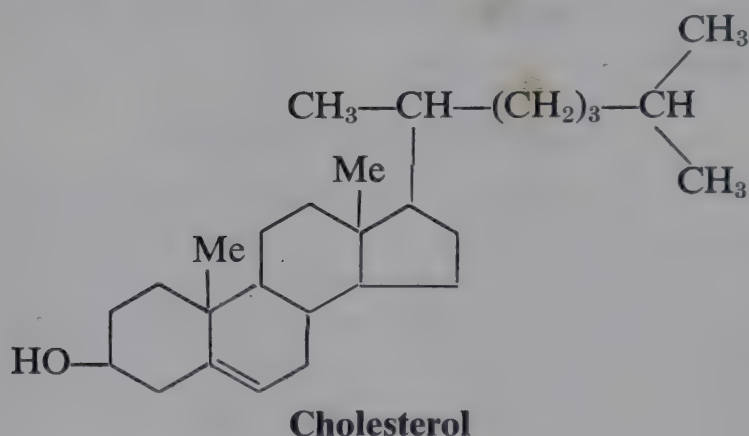
active ones in that they had a free hydroxyl group at C3, double bond between C5 and C6, and possessed a side chain.

With these findings under consideration, Fisher then determined the ability of these sterols to inhibit the hemolytic action of digitonin. Ponder (1945) had shown that the active point of digitonin fixation in the steroid molecule was the C3-C5-C6 region and Fisher found that the same sterol units which prevented hemagglutination were effective inhibitors of hemolysis by digitonin.

As previously mentioned the hemagglutination by influenza virus resembles that by *Hemophilus pertussis* supernatant. From the inhibition standpoint, however, the two are quite different. For example, the steroid inhibitors of the *Hemophilus pertussis* hemagglutination are ineffective against that of influenza virus and conversely the ovarian cyst polysaccharide which inhibits the virus hemagglutination is inactive against the pertussis reaction (Fisher, 1948). Again, erythrocytes treated with the receptor destroying enzyme of *Vibrio comma* remain agglutinable by pertussis supernatant but not by that of influenza virus.

Influenza Virus

Hirst (1942) from his studies of influenza virus adsorption on red cells concluded that the reaction between the virus agglutinin and the receptor at the cell surface was that of an enzyme-substrate interaction, and that the enzyme possessed by the virus changed the properties of the cell receptor



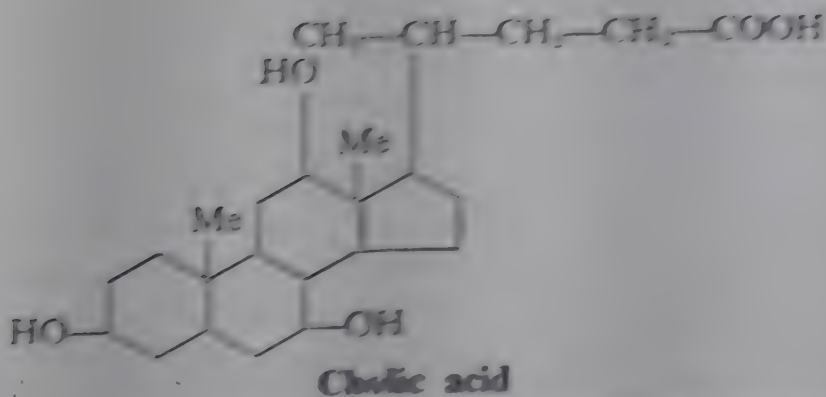
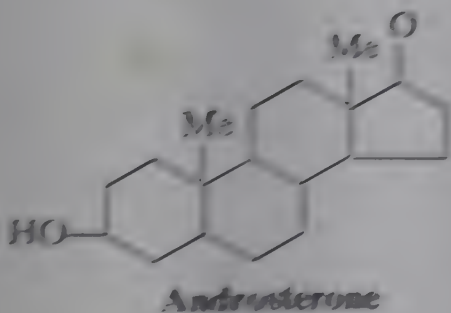
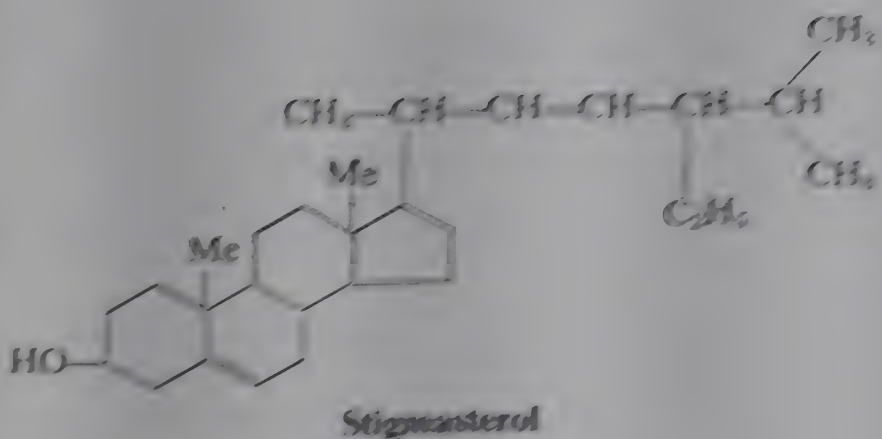
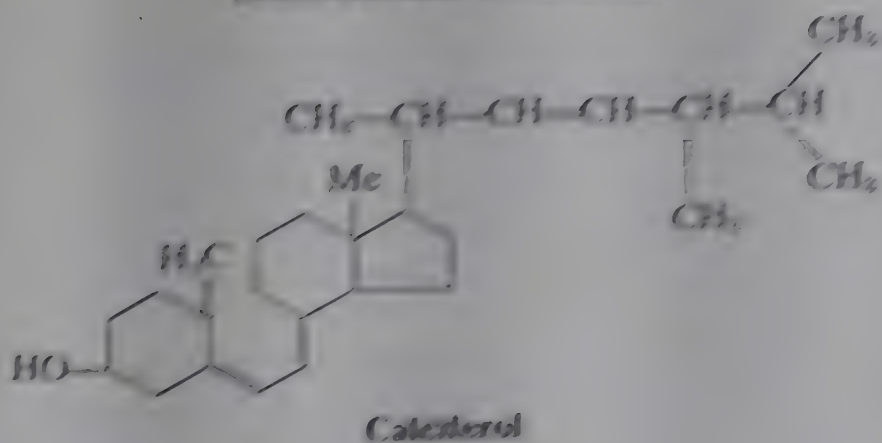


FIG. 8. Steroids tested as inhibitors of pertussin hemagglutinin.

substance. This conclusion was based upon his observation that following the elution of the virus from the cell surface, the red cell was irreversibly changed and could no longer adsorb virus particles.

Following Hirst's report, a series of papers appeared recording the power of normal human serum to inhibit hemagglutination by heated influenza B virus (Francis, 1947), the ability of mucoprotein from human serum and of a purified mucopolysaccharide from ovarian cyst fluid to function in the same manner (McCrea, 1948), and finally the capacity of a polysaccharide fraction from human red cells (Hirst, 1948) to block the reaction. These agents all represent substrate or enzyme displacers. It is probable that those of purely polysaccharide nature are substrate displacers and that those of protein nature such as the ovomucin inhibitor derived from hen egg white (Gottschalk and Lind, 1949) are enzyme displacers. The power of the ovomucin to inhibit the reaction is destroyed or reduced by influenza virus, by the receptor destroying enzyme of *Vibrio comma* and by trypsin. The implication is that the enzyme of the virus may well be proteolytic in nature. Its substrate would be a protein with a polysaccharide hapten as determinant grouping.

If the hapten of the protein substrate is a polysaccharide, then it is to be anticipated that the polysaccharide itself would act directly on the virus inasmuch as the enzymatic composition of any unit of living matter must be associated with the vitality of that particle. The observations of Green and Woolley (1947) fit into this pattern. They found that the complex carbohydrates from apple pectin, citrus pectin, flaxseed mucilage, blood group A substance, gum acacia, and gum myrrh, as well as an extract of chicken red blood cells, inhibited the agglutination reaction of chicken red cells and influenza A virus. The action of apple pectin also inhibited the multiplication of influenza A virus in embryonated eggs. These investigators interpreted their findings as a manifestation of competition between structurally similar substances. While they point out that displacement may not underlie all of the observed phenomena, it is equally true that none of their discoveries lies outside of the pattern of biological antagonism. For example, alginic acid was relatively inactive as inhibitor of both virus multiplication and hemagglutination reaction. On the other hand, the fact that some of their hemagglutination inhibiting carbohydrates (citrus pectin) did not reduce multiplication of virus can be interpreted as a manifestation of minor but significant reaction site structure differences on the red cell surface carbohydrate and on that of the normal internal virus substrate. None of the simple carbohydrates, i.e., galactose, galacturonic acid, etc., possessed the power of inhibiting the hemagglutination reaction.

This path to possible chemotherapeutic agents differs in no way from the usual approach to the creation of new antibacterial or antiviral agents through the use of molecules capable of interfering with their normal

metabolism, except in the fact that here the substrate is macromolecular. Study of the effect of polysaccharides on *Escherichia coli* phage led Maurer and Woolley (1948) to the finding that while these agents were neither bactericidal nor virucidal, they did prevent the attachment of the virus to the cell. The bacterial cells were protected from the lytic action of the phage by citrus pectin but not by gum acacia, starch, and other polysaccharides.

In further studies, Woolley (1949) demonstrated a substance in erythrocytes which antagonized the inhibiting effect of apple pectin on influenza virus hemagglutination. This can be interpreted as a manifestation of competitive antagonism in the sense that the substance isolated may have been identical with or similar to the polysaccharide contained in the surface of the red cell with which the virus normally reacted, and the result was that of added substrate overcoming the blocking action of substrate displacer.

Again, Ginsberg et al. (1948) have isolated from type-specific Friedländer bacilli a polysaccharide capable of inhibiting the multiplication of the mumps virus in the allantoic sac of the chick embryo. They established the nonidentity of the polysaccharide conferring type specificity on the bacterium and that causing inhibition of the mumps virus. As in the studies of Green and Woolley (1947), this group found that polysaccharides which cause inhibition of the multiplication of virus may or may not interfere with the inhibition of hemagglutination phenomena. The same variation in polysaccharide activity was seen in studies of virus adsorption by erythrocytes.

Recently, Ashenburg et al. (1950) have concluded that nonspecific inhibition of bacteriophagy by bacterial and nonbacterial polysaccharides is not necessarily contrary to the theory that susceptibility to lysis is related to antigenic structure. All of the polysaccharides they tested, including starch, glycogen, gum arabic, and certain homologous and heterologous capsular bacterial polysaccharides, inhibited the lytic action of the phage. The degree of this inhibition varied with the polysaccharide and with the host organism.

Ginsberg and Horsfall (1949) offered four potentialities in explanation of the inhibition of mumps virus by the polysaccharide of *Klebsiella pneumoniae*. The carbohydrate might inhibit (1) intracellular metabolic processes essential to the virus, (2) virus-cell combination, (3) release of virus from the cell after multiplication, or (4) invasion of the cell by the virus. Actually, all of these seem tied to the same general pattern. Permeability of the bacterial cell is associated with enzymatic activity of hyaluronidase and the degree of polymerization of the complex polysaccharide, hyaluronic acid. Any modification in permeability dependent as it is upon polysaccharide function in the surface structures would doubtless modify all four processes. Contact of phage carried enzyme with substrate would be altered in a similar manner. Permeability modification is a phenomenon which must

be taken into consideration in the interpretation of any studies of biological antagonism but it should also be held in mind that permeability alterations are equally the result of antagonistic systems.

It seems reasonably sure that virus reactions are intimately associated with the enzymes carried in the virus particle and the effect those enzymes have on substrates which may be and generally are components of the cell, whether that cell is bacterial or tissue. Structural analogues of the normal substrate can interfere with and block multiplication of the virus and its effect on the host cell.

General

Pauling's concept of the process of formation of antibodies is based upon the probability that the biological forces at play are those ordinary short-range ones known to exist between simpler molecules (Pauling, 1940). He assumes that the specificity of antigen-antibody relationships is due to a detailed "complementariness" in configuration over a considerable portion of the surfaces of the two molecular units. The multivalence of antigens and antibodies is also incorporated into the theory. The coiling of long chain polypeptide units in accordance with the catalytic effect of environment determines whether the chain will form normal γ -globulin or specific antibody. In this system, the enzyme or catalyst is the antigen and the substrate is the long chain polypeptide with the antibody corresponding to the product of the reaction. The entire hypothetical structure can be integrated into the theory of biological antagonism. The single added feature would be that of a substrate other than that catalytically converted into the antibody, which would be the host-provided substrate. The modification of this second substrate would in general account for the toxicity of the antigen. Structural features would be held in common by antibody and normal receptor substrate for the antigen.

The multivalence of antigens and antibodies accounting as it does for serological precipitation and agglutination (Pauling et al., 1944) has been substantiated by observations which are clearly manifestations of antagonism. Pauling et al. (1942, 1943) used antisera homologous to the p-azobenzenearsonate ion group and found that of the substances tested those containing two or more p-azobenzenearsonate ion groups in the molecule produced precipitates with the antisera, whereas those with only one benzenearsonate ion group did not form precipitates but rather inhibited precipitation of the antisera by the corresponding antigen. The assumption here is that the monohaptenic unit will combine with only one antibody molecule and that the resulting complex is not of sufficient size to precipitate. On the other hand, the polyhaptenic molecules will combine with two or more antibody particles and the aggregate will precipitate. While in this instance the simple haptenic unit forms the linkage of antibody molecules,

it is easy to translate the finding into the system where the antibody by virtue of its multivalency forms the cross linkage or framework onto which are attached antigenic units in sufficient number to create a macromolecular structure which precipitates.

Potentialities for multivalent antagonists are reflected in the existence of multivalent simple haptens in which the hapten units are not identical. Pauling et al. (1944) used these units to demonstrate the involvement of several haptenic groups in a precipitate formation. In the experimental design, antisera were developed in the blood of rabbits either to an azo-protein containing p-azobenzenearsonate ion groups or to one containing p-azobenzoate units. Then molecules were synthesized which contained both groups. These molecules were 1-amino-2-p-(p-azobenzeneazo)-benzenearsonic acid-3,6-disulfonic acid-7-p(p-azobenzeneazo)-benzoic acid-8-hydroxynaphthalene, and 1,8-dihydroxy-2-p-azobenzenearsonic acid-3,6-disulfonic acid-7-p-(p-azobenzeneazo)-benzoic acid-naphthalene. Neither of these substances gave a precipitate with one antisera alone but if mixed with both a heavy precipitate was formed. The probable explanation of this phenomenon was that the bivalent simple haptenic molecules united with antibody particles specific for both the azobenzenearsonate and azobenzoate ion groups. With two antibody particles, the complex formed was of sufficient size to precipitate, whereas if only one antibody unit reacted there was no precipitation.

The entire concept of biological antagonism would have had to be modified if the position of Rothen (1947, 1948) had been substantiated, for he proposed specific reactions between antigens and antibodies and between enzyme and substrate protein even when the units were separated by distances of hundreds of angstroms. Rothen's concept was in direct conflict with that of Pauling who indicated that short-range interactions were dominant as biological forces. The experimental basis of the work of Rothen was associated with reactions of proteins in thin films separated by thin inert barriers. Singer (1950) has found that the barriers used by Rothen actually have holes in them, permitting the macromolecules to come into contact and react. It would seem highly probable that the concept of Pauling will hold and that Rothen's observations were based upon inadequate experimental technics.

It is interesting to consider briefly the basic mechanisms of action of toxins, particularly in relation to enzymes and normal tissue substrates. In this connection, Pappenheimer and Hendee (1947) have confirmed the original observations of Fujita and Kodama (1934) who determined that the major iron porphyrin containing pigment of the diphtherial cell is related to cytochrome-b. Cytochrome-b and diphtheria toxin are probably related in the sense that diphtherial cytochrome-b is a pigment composed of iron, porphyrin, and diphtheria toxin. From this conclusion, Pappen-

heimer and Hendee (1947) logically assume that the action of diphtheria toxin would be that of interference in some manner with the function of cytochrome-b or related enzyme in the tissues of susceptible cells. Later, Pappenheimer (1948) specifically designated the action of the diphtherial toxin as directed against the normal function of cytochrome-b in the tissues of susceptible animals through blockage of the synthesis of this important moiety.

In this same line, the action of tetanus toxin has been proposed by Ambache et al. (1948) to be associated with the sensitization of muscle to cholinergic drugs. They base this assumption on experiments demonstrating the difference between the normal and tetanus toxin injected eyes of rabbits. The iris of the intoxicated eye underwent myosis with doses of carbaminoylcholine which were insufficient to produce pupillary contractions in the control eye.

Similar studies were conducted by Torda and Wolff (1947) with botulinus toxin. They used the Types A and B toxins and observed a decreased synthesis of acetylcholine when either of these materials was present. The responsiveness of striated muscle to acetylcholine was not modified by low concentrations of the toxins but was increased by higher concentrations. They propose that the "functional paralysis" observed in animals affected by the toxins of *Clostridium botulinum* is due to decreased acetylcholine synthesis, specifically related to myoneural function. Inhibition of acetylcholine synthesis is also a characteristic function of tetanus toxin (Ambache et al., 1948). The toxin apparently interferes with enzymatic function and does not affect the substrate.

Chemical antagonists of the action of botulinus toxin are known (Miller and Nelson, 1948) and were discovered on the basis of failure of nerve impulse transmission and attempts to reestablish this transmission chemically. Acetylcholine and various quaternary ammonium halides could not be used because no practicable tolerated doses were found under the experimental conditions. Both ammonium 2-nitro-1-butanefulfonate and 2-(hydroxymethyl)-2-methylpropylamine HCl were effective as inhibitors of the lethal action of botulinus toxin.

Interference Phenomena Among Viruses

Viruses are infectious agents, possessing the ability to reproduce but only in the living host cell. The viruses obtained from plant sources are nucleoprotein in character. Those from animals are more complex, having a composition which may approach that of a bacterium. The plant viruses generally have the pentose type of nucleic acid and in the animal virus the desoxypentose type is present.

The molecular weights of various viruses have been calculated from sedimentation rate studies. Tobacco mosaic has a molecular weight of 40

million and alfalfa mosaic around 2 million. In common with genes and antigens, it is probable that virus particles are autocatalytic in nature. Sevag (1945) has stated the probability that since all proteins are antigenic and all antigens are autocatalytic, all proteins must be endowed with enzymatic activity. Certainly, the hapten finds its counterpart in the coenzyme and in those structures associated with the virus particle which can be chemically modified *in vitro* with resultant biological alteration. It is well to consider at all times the probability that enzyme, virus, gene, and antigen are but manifestations of the catalytic character of protein structures with specificity determined by hapten-like moieties.

The hapten or cofactor of a given protein molecule can be considered to be the amino acid moiety which is not intimately linked into the peptide chain. It is the R of the standard type structure, $R-CH(NH_2)-COOH$. Amino acid analysis of eight different strains of tobacco mosaic virus have been made by Knight (1947) who reports profound differences. One strain was found to contain histidine and methionine, amino acids not present in the other seven strains. In general, he found the greatest differences between strains whose biological properties would suggest that they were most remotely related. The reverse was also true: that differences in composition in closely related strains were slight. In one case, an immediate mutant strain differed from the parent in only two respects. Thus, it is apparent that protein structures within the virus complex differ qualitatively, a point distinguishing them from antibodies.

Polysaccharides play a significant role in specificity manifestations with viruses, just as they do with antigenic materials in general. For example, polysaccharides from type-specific Friedländer bacilli inhibit the multiplication of mumps virus and may also inhibit hemagglutination caused by the virus (Ginsberg et al., 1948).

Anderson (1948) has suggested that the existence of a cofactor for the activation of T4 bacterial virus resembles that seen with enzyme systems. L-Tryptophan, the cofactor in this case, makes possible the adsorption of the virus particle onto the host cell. The function of the tryptophan may be that of a bridge from virus particle to cofactor; this might be a component of the host cell or it might confer upon the particle the structural characteristics essential for the degree of complementariness necessary to the function of autocatalytic proteins in general. While tryptophan is the most active amino acid, phenylalanine, tyrosine, and certain tryptophan analogues also possess this power. Again, the capacity of indole (Delbrück, 1948) to inhibit the adsorption of phage particles parallels to a considerable degree the metabolite displacement phenomena so common to enzymatic reactions.

In the consideration of interference phenomena in the study of viruses, the antiphage or antiviral body used prophylactically is not excluded since

it directly obstructs the key-to-lock relationship of virus to susceptible host cell. The active, the attenuated, and the fully inactivated virus can be used to induce immunity. The major factors in acquired immunity are the antibodies found in the γ -globulin fraction of blood proteins. These antibodies are capable of neutralizing virus activity, agglutinating the respective antigens in some case forming precipitates with the soluble antigens, and finally fixing complement (Burnet et al., 1937; Craigie, 1939). Viruses are therefore antigens in the usual sense and statements made concerning antigens and their activity apply equally to the viral antigen.

There is one instance of record in which an enzyme inactivated a virus. The enzyme was crystalline d-ribonuclease which formed a combination with tobacco mosaic virus causing a reversible inactivation of the virus (Loring, 1942). The virus-enzyme complex could be dissociated, liberating the virus in a fully active form. The dissociation could be caused by diluting the solution of the complex, sedimenting at high speed, and redissolving the pellets.

While all of the aforementioned factors enter the picture of the antagonistic activity associated with the virus, we are primarily concerned with the capacity possessed by certain viruses to interfere with the growth and effect of other viruses. There are instances of this type with which one virus will interfere with the second but the second will not block the first. Here we have biological antagonism associated with "living" particles, with molecular complexes capable of reproducing themselves.

Interference was first noted between plant viruses (McKinney, 1926). Work in this field has been reviewed by Price (1940). Interference appears to be limited to viruses which are closely related and can even be used for purposes of differentiating and classifying these agents.

Interference phenomena between animal viruses has recently been extensively reviewed by Henle (1950). As this review is comprehensive, we here repeat only the material necessary for the incorporation of this interference field into the general concept of biological antagonism.

Hoskins (1935) reported the first extensive investigations into the interference aspects of animal viruses. He found that the neurotropic strain of yellow fever virus protected against the viscerotropic strain of the virus. His observations were confirmed (Findlay and MacCallum, 1937; Lennette and Koprowski, 1946). Similar studies have disclosed the opposing effects of nonencephalitogenic and encephalitogenic strains of herpes simplex virus (Doerr and Kon, 1937; Doerr and Seidenberg, 1937), and neurotropic and pneumotropic influenza viruses (Andrewes, 1942). In these instances, the virus strains vary in their tropism.

The work of Lennette and Koprowski (1946) was done in tissue culture. They found that the 17DD strain of yellow fever virus was capable of suppressing completely the growth of both the Asibi strain of the same virus

and the heterologous West Nile virus. Furthermore, both the 17DD strain of yellow fever virus and the West Nile virus produced partial or complete suppression of the growth of the Venezuelan equine encephalomyelitis virus and of the influenza A virus. The possibility of cultures being contaminated by neutralizing antibodies or nonspecific antiviral agents was ruled out.

Again, interferences are seen between neurotropic viruses. For example, Rhodes and Chapman (1949) using experimental animals found no evidence that the inoculation of animals with human poliomyelitis virus interfered with the growth or effect of the MM or Lansing strains of this virus, but they did see interference between lymphocytic choriomeningitis virus and the MM virus. The reverse also held: under certain circumstances inoculation with MM virus may lessen the severity of the illness due to earlier injection of lymphocytic choriomeningitis virus.

As another example, Vilches and Hirst (1947) observed the interference reaction between the viruses of influenza A and B on the one hand and that of western equine encephalomyelitis on the other when intracerebral inoculations were made. Using the intraperitoneal route for administration of the influenza virus, the antagonism with encephalomyelitis was not evidenced. Furthermore, the interference could not be demonstrated in the allantoic cavity of the embryonated hen's egg.

In addition to the interferences occurring between plant and animal viruses, there are instances of such reactions between bacterial viruses or phages. Delbrück and Luria (1942) discovered the antagonism of the $\alpha(T_1)$ phage of *Escherichia coli* for the $\gamma(T_2)$ phage of the same microorganism. Further, the ultraviolet light-inactivated $\gamma(T_2)$ phage retained its power to suppress the growth of $\alpha(T_1)$ phage (Luria and Delbrück, 1942). Another interesting finding was that the inactivated $\gamma(T_2)$ phage interfered with the growth of $\gamma(T_2)$ phage.

From consideration of all studies of interference between two active viruses, there are certain generalizations that can be made. The phenomenon can occur in an extremely broad spectrum of host animals including calves, chickens, guinea pigs, mice, hamsters, foxes, possibly man; and tissues including central nervous system, respiratory tract, skin, cornea, etc. There is no restriction of the interference phenomenon to certain types of viruses. They range from the largest to the smallest, from the fully pathogenic to the mild and even to the agent producing an infection detectable only serologically.

Another generalization is that the interfering agents may precede the excluded virus, may be given simultaneously or may follow and in each case the basic mechanism can be demonstrated. Again, while in most cases interference is reciprocal, there are some in which no reciprocity has been found. Still another important fact in the consideration of this type of biological antagonism is that it is not universal. There are instances of dual

viral infections and these may occur within the same cell. Finally, interference reactions are distinct from those of specific immunity reactions. This last statement is based upon the rapidity of the appearance of the interference phenomenon and on its occurrence between agents which do not possess common antigenic components.

With two active viral antagonists, complications arise which can be eliminated if the interference is caused by an inactivated virus. The inactivation can be brought about by heating, formalinization, and ultraviolet light. With the inactivated particle, the interference is linked directly to the unit and cannot be separated therefrom. Another factor for consideration is that the interfering power is due to particular groupings on the particle which are distinct from those involved in other viral activities.

Our purpose is the integration of the facts of viral interference phenomena within the framework of biological antagonism and we are therefore fundamentally concerned with the mechanisms of this reaction. There are three hypothetical resolutions of this problem: the penetration concept, the theory of blockage or destruction of cell receptors, and the hypothesis of competition for an enzyme system.

The first of these, the penetration concept, assumes that the entrance of one bacteriophage particle into the host cell so alters the cell surface as to prevent the penetration of a second type particle (Delbrück, 1945). This concept has been largely abandoned following the demonstration of dual infections of bacterial cells.

The second theory, that of blockage or destruction of cell receptors, is based largely upon studies of hemagglutination which were conducted by Hirst (1942). He found that red cells adsorb influenza virus and are, as a consequence, agglutinated. Subsequently, the virus can be eluted from the red cells and after this elution, the red cells no longer possess the power for adsorption of the virus. The virus particle itself is unchanged. In general, the additional supporting evidence follows similar lines and has been previously reviewed.

While interference on the basis of receptor destruction may well play a part in the phenomenon, there is evidence indicating that the major role is assumed by another mechanism. One aspect of this evidence is the fact that influenza virus inactivation by ultraviolet light shows loss of interfering capacity by the virus within ten minutes, while the adsorption mechanism is unaltered after 60 minutes (Henle and Henle, 1947). Another factor indicating a mechanism other than that of receptor destruction is the finding that cells which have been rendered resistant by induction of the interference reaction still adsorb additional virus of either the homologous or heterologous types (Ginsberg and Horsfall, 1949a; Henle et al., 1947).

Still another line of evidence tending to eliminate the receptor destruction concept as the key mechanism underlying the interference phenomenon is

the observation (Florman, 1948) of reciprocal action of influenza and Newcastle disease viruses with regard to interference but not to adsorption elution effects. Then, Schlesinger (1949) added the final bit of evidence against receptor destruction when he observed that the viruses of equine encephalomyelitis do not destroy the receptors for influenza virus in the brain of mice. Interference between these two viruses has been demonstrated (Henle and Henle, 1945; Vilches and Hirst, 1947).

The enzyme competition hypothesis for the interference reaction which was elaborated by Delbrück and Luria (1942) has come to be accepted. Henle (1950), having reviewed the entire field in some detail, concluded that this was the "most plausible." According to this concept, the viruses compete within the host cell for an enzyme system essential to the propagation of either one of them. The enzyme system involved might be a part of the invading virus or it might be a component of the host cell. It is also true that there is no reason to restrict the inhibitory action to a single enzyme, as it may with equal probability affect many enzyme systems. The displacement phenomena would be that involving the protein moiety of an enzyme; however, the hapten inhibition angle must be considered a possibility.

Substantiation of this concept takes several lines. The first of these attempts to establish the reactions as intracellular by proving the failure of specific antibodies to attack a virus after the virus has had a chance to establish itself within the host cell (Henle and Henle, 1949). This is interpreted to indicate penetration of virus into the cell, a power not possessed by the antibody. Another experimental finding lending support to this position is that the interfering action of irradiated and inactivated virus is not altered by subsequent treatment of the system with immune serum (Henle et al., 1947).

The second line of investigation tending to support the enzyme displacement concept reveals the effect of the inhibitory virus on the metabolism of the host cell as reflected in its ability to block further development of allantoic sacs used as test structures (Henle et al., 1947) and to alter the metabolic activities of bacterial host cells (Cohen and Anderson, 1946).

In conclusion, it can be stated that the enzyme-inhibition concept of virus interference reactions seems the most logical and is supported by ample investigational evidence. It indicates the fact that complexity is no reason to eliminate competitive or noncompetitive displacement phenomena from consideration in studies of biological antagonism.

Protein Enzyme Inhibitors

The preceding consideration of antagonisms as manifested by immunological reactions and virus interferences might well be considered special

classifications of protein enzyme inhibitors and as such included in this section. The separation is for convenience of presentation and does not imply distinction.

Zymoids

One class of protein enzyme inhibitor is the zymoid (Bearn and Cramer, 1907), a denatured enzyme. Solutions of enzymes which have been heated to 56°–60° C. for 20–30 minutes have a strongly inhibitory effect on the activity of the unheated enzyme. Heating to 100° C. destroys the antagonistic effect. The phenomenon is closely related to the production of heat-inactivated virus particles and the capacity of these units to interfere with the working of the active virus. It would seem logical to anticipate alteration in surface configuration by heating (denaturation) and if this modification were of minor degree displacement phenomena should ensue between the native and the denatured forms of any protein.

Enzyme Inhibiting Immune Bodies

The protein enzyme inhibitors of this group are manifestations of immunological reactions. They are produced by injecting an enzyme parenterally. Antibodies have been described against emulsin, amylase, invertase, urease, rennin, pepsin, trypsin, phenolase, lipases, etc. In fact, theoretically it is possible to develop antibodies against any enzyme.

The subject of the enzyme as an antigen and of the corresponding antibody as a specific enzyme inhibitor has been reviewed in detail by Sevag (1945). For the purposes of summarizing the subject for the consideration of these reactions as biological antagonisms, it will be necessary to present only a few selected instances.

The proteolytic enzymes offer a pathway into the maze of observations in this field. Northrop (1930) first demonstrated that crystalline pepsin protein gives rise to pepsin precipitating antibodies. He used both active and inactive pepsin and these antigens gave rise to antibodies which gave cross-reactions. This was to have been anticipated as pepsin is inactivated above pH 6; and in even more alkaline media such as that of the blood stream (pH 7.6) the pepsin molecule is denatured. Antibody formation is therefore to the denatured form rather than to active pepsin. The design of the experiments of Seastone and Herriott (1937) was based upon the denaturation and they used as antigens alkali-inactivated pepsins. They were able to distinguish serologically among the pepsins of several different animal species. The unfortunate aspect of the pepsin-antigen-antibody system is that there is no way of demonstrating the inhibition by the antibody of the enzymatic activity of the pepsin.

With trypsin, the situation is different and permits of demonstrating enzymatic activity reduction or blockage. TenBroeck (1934) using the anaphy-

lactic reaction in guinea pigs found that chymotrypsin and trypsin differ in their properties. The original demonstration of trypsin antibody as an inhibitor of the activity of the enzyme was made in 1901, years before any work was carried out with purified enzyme preparations. Achalme (1901) observed the toxic action of a trypsin preparation in guinea pigs. He attributed the toxicity to the effect of the enzyme. To prove the point, he then immunized guinea pigs with the trypsin preparations and produced a serum which inhibited the tryptic activity of the enzyme *in vitro* and completely protected the guinea pigs against its toxic action.

It is probable that the degree of complementariness which must exist between antigen and antibody and between enzyme and substrate causes in this case similarity of structure between antibody and substrate. The displacement might result either from a combination of enzyme and antibody or of antibody and substrate. Quantitative considerations immediately eliminate the antibody substrate combination concept and leave the probability of a competition existing between antibody and normal substrate for structural reactive surface position on the enzyme-antigen unit.

Papain is another example of a proteolytic enzyme having antigenic capacity for the elicitation of antibody formation with subsequent inhibition of its own effect. Haas (1940) made the observations which concerned the inhibition *in vitro* of the proteolytic hydrolysis of casein or gelatin. The experiments were carefully controlled. Reversibility of the enzyme-antibody complex was established by leaving the precipitated complex in the system and finding evidence of partial hydrolysis.

Rennin is a milk coagulating enzyme. It converts casein to paracasein and if there is enough calcium present the paracasein separates out as a gel. With crude preparations of this enzyme from plant and animal sources, Morgenroth (1899, 1900) was able to prepare an immune serum with the capacity to prevent the action of rennin. In the presence of the proper amounts of the antisera, rennin did not coagulate milk. The findings of Morgenroth were confirmed by Thaysen (1915), although the latter investigator interpreted the results differently.

The first unquestionable demonstration of the development of antibodies following the injection of enzymes resulted from the work of Sumner and Kirk (1931) and Kirk and Sumner (1931, 1932, 1934) with crystalline urease. The antibody obtained gave the usual immunological reactions with urease and prevented *in vitro* the urease catalyzed conversion of urea to ammonium carbonate. Further it protected animals against the toxic effects of urease injection, associated with the rapid formation of ammonia. Urease inactivated by Formalin is both nontoxic and incapable of stimulating anti-urease-antibody formation. With urease and its antibody and substrate, we have an example of a system in which a simple molecule, urea, functioning as substrate, competes with a macromolecular unit, a glycoprotein which

is the antibody, for a position on the surface of an enzyme. Molecular magnitudes need not match for inhibitory phenomena to be manifested.

Still another example of the development of antibodies to enzymes concerns catalase, an iron-containing enzyme primarily involved in the conversion of hydrogen peroxide to water and oxygen. Tria (1939) and Campbell and Fourt (1939) found the development of an anticatalase following the injection of crystalline beef liver catalase. The anticatalase was serologically identified by precipitation reactions. Biochemically, the question immediately arises as to whether or not the catalase has lost its activity. With the proteolytic enzymes and with urea, enzymatic power is completely nullified by the corresponding antibodies, but with catalase the situation is questionable. Campbell and Fourt (1939) observed very little loss of activity but their experiments involved dilution factors and the potentialities for dissociation of the complex were great. Again, Harkins et al. (1940) using a different technic (multilayer) also reported enzymic activity in the catalase-anticatalase complex. While the observed activity was only one-fifth to one-tenth that of the enzyme in solution, they felt that denatured protein or impurity might tend to give results indicating such losses.

Catalase is a metallo-protein enzyme and the possibility arises that this enzymatic type will in general not be inhibited by the corresponding antibody. The question remains unsettled. Tyrosinase, a copper protein enzyme, has been found (Gessard, 1901, 1902, 1903, 1906) to be inhibited in its action by the proper antibody. Bach and Engelhardt (1922), using mushroom extract, confirmed these findings; but Adams (1942) claims that the antibody has no effect on the catalytic activity of the enzyme.

Again, a striking substantiation of the probable general inhibition of enzyme activity by antibody is the finding of Harvey and Deitrick (1930) that an antienzyme could be developed against luciferase and that this anti-enzyme prevented luciferin from luminescing when it was mixed with luciferase.

As a final example, it seems suitable to use hyaluronidase, the permeability factor. In 1936, McClean observed that the hyaluronidase activity of culture filtrates of *Clostridium welchii* was not completely inhibited by an amount of antitoxin sufficient to neutralize the toxin. Normal horse serum had no inhibitory capacity. The combined findings led McClean to propose the existence of an antibody for the hyaluronidase (permeability factor). Later, McClean (1941) using filtrates of *Clostridium oedematis maligni* cultures produced an antisera to hyaluronidase which prevented the action of the enzyme on hyaluronic acid. Similarly, the antibody to the permeability enzyme in filtrates from cultures of *Clostridium septicum* blocked the liberation of N-acetylglucosamine from a mucoprotein substrate by the enzyme (McClean and Hale, 1941).

There are many other examples of the inhibitory action of antibodies for the enzymatic activities of the corresponding antigens, but in the interests of brevity these will not be listed. Sevag (1945) has listed all experimental findings in this category in a complete and comprehensive manner. The material presented establishes the ability of a specific antibody to block activity of enzymes and there seems no reason to regard this phenomenon in a manner distinct from that of any other competitive or noncompetitive enzyme inhibition.

Naturally Occurring Inhibitors

In considering the naturally occurring inhibitors of enzymes, we are restricting the case to proteolytic enzymes. There are many naturally occurring enzyme inhibitors. In fact, the products of any enzymatic reactions are in reality inhibitors thereof. For this reason, the heading of this section may seem ambiguous unless restricted as indicated.

The round worm, *Ascaris lumbricoides*, is an intestinal parasite, and its ability to withstand digestion by the proteolytic enzymes of the tract resulted in the initial investigations of natural antienzymes of this class. Weinland (1903) made extracts of these parasites and found that they were both antipeptic and antitryptic. Mendel and Blood (1910) confirmed these findings and extended them by discovering the failure of the extracts to inhibit papain. The nature of the inhibiting principle was delineated by Collier (1941) who found it to be a polypeptide.

During the course of the transformation of pepsinogen to pepsin (Herriott, 1938, 1941) and of trypsinogen to trypsin (Kunitz and Northrop, 1936), peptide inhibitors of the respective enzymes are formed. The peptides have a molecular weight of from 4000 to 10,000 and are specific, giving no cross-reactions for other proteolytic enzymes. The enzyme-inhibitor complexes are dissociable and seem to behave in accordance with the law of mass action. It is not surprising that inhibitors of protein enzymes of the proteolytic type are polypeptides. It would be most unusual if some antagonistic principle were not amino acid in structure.

It is even possible for enzymes to inhibit enzymes, as has been demonstrated by Sang (1938) who found that the *Ascaris* inhibitor of Weinland (1903) was in fact a protease. Another example of this type is the antagonism of urease for trypsin (Tauber and Kleiner, 1931). This interference phenomenon is unilateral in the sense that the union does not block urease action but does block that of trypsin.

The low nutritive value of raw soybean proteins led directly to the discovery in soybeans of a specific trypsin inhibitor (Ham and Sanstedt, 1944; Bowman, 1944). The soybean inhibitor when fed to chicks (Ham et al., 1945) or rats (Klose et al., 1946) caused retardation of growth. It is not certain that the inhibitor principle for trypsin and the growth retarding

principle are identical, but it seems probable. Kunitz (1945, 1946, 1947) has isolated the antagonistic factor and found it to be a globulin type protein. He observed a loss in amino nitrogen when the combination of trypsin and inhibitor occurred which indicated a reaction of the amino groups of the enzyme with the carboxyl groups of the inhibitor.

Similarly, a crystalline trypsin inhibitor has been isolated from ovomucoid (Lineweaver and Murray, 1947) which behaves in much the same manner as does the soybean antagonist (Fraenkel-Conrat et al., 1949), in that amino groups of trypsin and carboxyl groups of ovomucoid are required for the reaction.

Still another type of inhibitor, differing from that isolated from soybean and ovomucoid, has been obtained from lima beans (Fraenkel-Conrat, 1948). As with the soybean factor, the lima bean trypsin inhibitor radically interferes with the growth of Swiss mice when fed at a level of 1.7 per cent of the diet (Tauber et al., 1949).

It is probable that the antibacterial effects of certain protamines and histones (McClellan, 1931; Miller et al., 1942; Woolley and Krampitz, 1942) as well as their virucidal (McClellan, 1930) and fungicidal properties (Stuart and Harris, 1942) are attributable to similar enzymatic blockage phenomena.

These naturally occurring polypeptide and protein inhibitors of proteolytic enzymes fall into the general classification of macromolecular biological antagonists. They are structural analogues of the enzymes they inhibit. As there are displacers for substrates of enzymes and for cofactors of enzymes, so there are displacers for the entire protein moiety of the enzyme itself.

Antagonisms of Protein or Polypeptide Hormones

There are two phases to the consideration of antagonisms of protein and polypeptide hormones. One concerns the antigenic power of these agents and the other the mutual interference of one hormone for a second.

The behavior of protein hormones as antigens was first observed by Collip and Anderson (1934) in their studies of the sera of animals refractory to the action of certain pituitary hormones. They observed that the serum of a thyrotropic hormone resistant animal would prevent the action of the hormone on a normal, nonresistant animal. The term "Antihormone" was created to designate the antibody concerned. Collip initially did not regard the antihormone as an antibody.

Thompson (1941) has reviewed the subject of antihormones in some detail and it is our intention to do no more than refer to a few key articles as the basis for the integration of this group of antagonistic principles into the general concept of biological antagonism.

Shortly after the appearance of the work of Collip and Anderson

(1934), other investigators established similar antihormones for ketogenic (Black et al., 1934; Black, 1935), growth (Collip, 1935), adrenocorticotrophic (Thompson and Cushing, 1934), and the gonadotropic hormones (Selye et al., 1934) of the anterior pituitary, and for the anterior pituitary-like hormone of the urine of pregnancy (Bachman et al., 1934).

In view of the current extensive use of adrenocorticotrophic hormone and cortisone in the treatment of rheumatoid arthritis and similar diseases, it is of particular interest to note that both the polypeptide (Hartman et al., 1937) hormone and one of the corresponding steroid hormones, corticosterone (Hartman et al., 1940) produce antihormones under certain conditions. In the latter instance, the steroid functions as a hapten moiety.

The physiological effects of antihormones correspond in general to the effect of defective hormone formation syndromes. As an example, the antihormone for the thyrotropic agent causes the basal metabolic rate of rats to decrease below the normal level (Anderson and Collip, 1934). Furthermore, the tendency of the thyrotropic hormone to raise the basal metabolic rate is blocked by the inhibitor. Again, the thyroid gland of the normal animal becomes atrophic under treatment with the antihormone (Eitel and Loeser, 1934). Similarly, the antihormone for the gonadotropic principle (Rowlands, 1937, 1939) inhibits ovulation and corpus luteum formation, causes atrophy of the reproductive organs, suppresses blastocyst implantation, and in general produces the effects on the reproductive system caused by hypophysectomy.

The phenomena under consideration seem no more than the manifestations of the antigen power of a protein hormone. The hormone functions in the strictest sense as an enzyme which in turn has the power of catalyzing the formation of antibodies. The antihormone or antibody is then a displacing agent preventing the reaction of the hormone in its enzymatic or hormonal function with its normal substrate. It should be emphasized that there are many who incline to the view that antihormones are formed as the result of contamination of hormone preparations (Evans and Simpson, 1950). Even if this were true, hapten function by the hormone would have to be conceded.

Aside from the antagonism of antihormone and hormone, there has accumulated ample evidence to establish the mutually antagonistic power of protein or polypeptide hormones. One example of the opposing effects of these hormones is the action of growth hormone and of adrenocorticotrophic hormone on nitrogen metabolism. The growth hormone causes a retention of nitrogen (Marx et al., 1942). On the other hand, ACTH causes weight loss and increased excretion of nitrogen (Evans et al., 1943; Moon, 1937; Noble and Collip, 1941). When the two hormones are given together, weight increase in the hypophysectomized animal is reduced or abolished

(Marx et al., 1943; Noble and Collip, 1941). The ACTH has apparently displaced the growth hormone from those receptor sites which normally form its substrate.

Another experimental design for the demonstration of antagonism between growth and adrenocorticotrophic hormone is that used by Becks et al. (1944), involving the osseous system. ACTH retards chondrogenesis and osteogenesis in the region of the proximal epiphysis of the tibia. In the hypophysectomized animal, ACTH can do nothing to the already inactive epiphysis; the growth hormone causes a resumption of activity. When the two are given together, the effects indicate antagonism as reflected in width of epiphyseal cartilage, endochondral bone formation, osteoblastic and osteoclastic activity, and regularity of cartilage columns.

The hypophysectomized animal exhibits hypersensitivity to insulin. This abnormal sensitivity is corrected by the administration of extracts of the anterior pituitary (Russell, 1938, 1942), an effect referred to as a "diabetogenic" action. Specifically, the diabetogenic factor of the anterior pituitary has been tentatively identified as the growth hormone (Shipley and Long, 1938; Young, 1939, 1945). It remained for De Bodo et al. (1950) to establish the growth hormone as the factor. In their experiments, the insulin hypersensitivity was completely abolished by this factor and concomitantly an impairment of glucose tolerance developed.

The mechanism of action of the growth hormone in producing a diabetogenic action has been considered by Anderson and Long (1947) who studied the elaboration of insulin by the isolated perfused pancreas. They found that insulin was normally secreted in response to elevated blood sugar levels. The growth hormone prevented the release of the insulin. As a manifestation of biological antagonism, this observation could be interpreted as being due to displacement by the polypeptide hormone of the glucose from a reaction site on the insulin molecule or on the receptor mechanism which stimulates secretion of the insulin.

ACTH produces a temporary diabetes mellitus in human subjects (Conn et al., 1948) which has been interpreted as due to an antagonism between insulin and ACTH for the peripheral biochemical reaction site of the insulin (Lotspeich, 1950).

Comment

The hypothesis is offered that proteins which occur alternately in fibrous and globular forms in accordance with functional activity will offer surface sites for the reaction of biological antagonists in accordance with the form. Reaction sites will vary with the functional state of the protein. Assuming these points as probable, it is logical to consider that such phenomena may underlie the trigger mechanisms for muscular contraction and relaxation. The physiological and biochemical implications of this concept are very

broad, i.e., a protein in globular form may be an enzyme of specific type while the same protein in fibrous form will possess no similar activity.

The details of protein structure and surface nature will form the basis of the future science of biological antagonism. It is after all the structural surface characteristics which determine reaction sites and therefore the degree of specificity seen in any inhibitory phenomenon. Coenzymes, substrates, and other correlatives of the protein enzyme moiety are but manifestations of the complementariness of reaction sites and reacting material.

Immunology is a subdivision of the science of biological antagonism. Antigens, including those of a toxic nature, are enzymatic in character. The toxic effect is due to the action of the antigen-enzyme on a substrate normally present in the host tissue, producing a toxic chemical. Another function of the antigen-enzyme is the elicitation of the formation of the antibody from serum globulins. The antigen-enzyme in this reaction is acting upon its substrate, the globulin of the serum, and the product of the reaction is the antibody. The antibody in the case of the toxin is no more than a displacing agent, preventing the action of the antigen-enzyme on a substrate which would be converted into a toxic chemical. The complementary structure of the antibody simply coats the antigen-enzyme, thus blocking the substrate reaction sites.

The artificial antigens prepared from simple hapten units unfolded worlds of information on biological inhibition. The simple hapten possessed the power of blocking the formation of artificial antigen-antibody precipitates, and this fact permitted the most intimate and detailed analysis of structure and biological specificity. In this connection, the theory is offered that the same physico-chemical forces form the basis of enzymatic inhibition by substrate analogues, of simple hapten interference phenomena and isomorphism in crystals. In addition, it can be stated that in general chemicals which in an enzyme system function as substrate and displacer will exhibit the hapten interference phenomenon if artificial antigens can be made with either and finally, will together form double crystals.

The proposition is restated that all proteins under the right conditions, in the proper environment or testing system, will function as enzymes. Further, antigens, viruses, genes, enzymes, and protein hormones represent the autocatalytic character of proteins and their specificity is determined by haptens whether these haptens be amino acid segments or nonamino acid in nature. The term, hapten, is in this sense equivalent to coenzyme, co-factor, etc.

The sphere of biological antagonism is not alone that of the simple molecules, the small units, functioning as substrates; it extends to the giant, macromolecules which may be said to possess "life." The interference phenomenon which is seen among the viruses is competitive inhibition on a scale involving molecules with molecular weights well into the millions.

Biological antagonisms are known between any of the following pairs of macromolecules: antigen-antibody, enzyme-enzyme, enzyme-virus, virus-virus, enzyme-antibody, denatured enzyme-enzyme. In addition, there is no necessary correlation between displacer and displaced substance as is seen in the displacement of urea from its reaction site on the urease surface by the corresponding antibody. The interpretation here is that competition is direct between urea and antibody for the reactive surface site on the enzyme.

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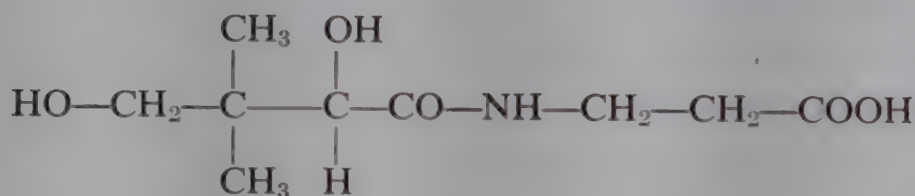
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Chapter 11

METABOLITE ANALOGUES OF PANTOTHENIC ACID



Pantothenic acid
(+)- α,γ -Dihydroxy- β,β -dimethylbutyryl- β' -alanide

1. Analogues with β -Alanine Moiety Modification, Including β -Alanine Analogues.
2. Analogues with Pantoic Acid Moiety Modification.
3. Analogues with Both Pantoic Acid and β -Alanine Moiety Modification.
4. Compounds Reported to Act by Virtue of Interference with Pantothenic Acid Metabolism.
5. In Vivo Studies.
 - a. Antibacterial.
 - b. Production of Pantothenic Acid Deficiency.
 - c. Antimalarial Studies.
 - d. Trichomoniasis.
6. Recapitulation.

Pantothenic acid is a growth stimulating factor, often called "the universal vitamin." It occurs in all types of animal tissues and is a general protoplasmic component. The vitamin is produced by various microorganisms and by green plants. Microorganisms are here classified as being of two types: those which synthesize the vitamin, and those which require an external source. Subdivisions in the latter category include those requiring the entire preformed molecule, those which need only β -alanine, and those which require the pantoic acid moiety.

In animal tissue, pantothenic acid occurs largely in a bound form, essentially as coenzyme A, which functions in acetylation mechanisms and it is probable that this is the major metabolically active form of the vitamin. The factor is essential for mice, rats, foxes, pigs, dogs, birds, sheep, cattle, and probably human beings, although the human requirements have not been established.

Chemically, pantothenic acid can be regarded as a peptide of β -alanine

and pantoic acid. Modifications in the molecule for the production of effective metabolite analogues might be affected by alteration of either the β -alanine moiety or the pantoic acid moiety or both. The free carboxylic radical suggests an obvious point of attack.

Analogues with β -Alanine Moiety Modification, Including β -Alanine Analogues

Pantoyltaurine, N-(α,γ -dihydroxy- β,β -dimethylbutyryl) taurine, was the first metabolite analogue of pantothenic acid with a β -alanine moiety modification synthesized and studied. Three groups—Snell (1941) in the United States, Kuhn and his co-workers in Germany (1941), and McIlwain (1942) in England, each working independently of the others—published their findings at approximately the same time.

Snell (1941) prepared pantoyltaurine, the sulfonic acid analogue, and found that it inhibited the growth of microorganisms requiring pantothenic acid. With those organisms capable of synthesizing pantothenic acid, the sulfonic acid analogue had no toxic action, nor was it toxic to organisms which could utilize β -alanine.

Kuhn et al. (1941) emphasized the variation in potency of the optical isomers of pantoyltaurine. With a constant concentration of pantothenic acid (0.05% of d-pantothenic acid per cc.) and increasing amounts of pantoyltaurine, about 100% per cc. of the d-pantoyltaurine sufficed to repress growth of *Streptobacterium plantarum* for 48 hours, whereas more than 3500% of the levo compound was required to produce the same effect. The growth concentration curves showed that the dextro compound was 25 times more potent than the levo pantoyltaurine. The probability of contamination of the levo compound by dextro was such as to suggest that the levo compound possessed no inhibiting power whatever. Some concentrations of β -alanine and α -hydroxy- β,β -dimethyl- γ -butyrolactone counteracted the effect of pantoyltaurine, which observation is in conformity with that of Snell (1941).

McIlwain (1942) had prepared three pantothenate analogues which were effective but had less affinity for the pantothenic acid enzyme reaction site than did the vitamin. The compounds were pantoyltaurine (Fig. 2) with antistreptococcal index of 500 (i.e., 500 times the concentration of pantothenate present was required to prevent the growth of streptococci) and an antipneumococcal index of 1000; pantoyltauramide (Fig. 2) with values of 100 and 10,000; and homopantoyltaurine (Fig. 2) which was less active. Pantoyltauramide and homopantoyltaurine were effective against all organisms susceptible to pantoyltaurine, and with the organisms tested (streptococci, pneumococci, *Corynebacterium diphtheriae*) the same order of susceptibilities held, with pantoyltaurine most inhibitory and homopantoyltaurine least. Barnett and Robinson (1942) had also prepared the

Table I

METABOLITE ANALOGUES OF PANTOTHENIC ACID IN
WHICH THE β -ALANINE MOIETY HAS BEEN MODIFIED

Compound	Activity	Reference
Pantoyltaurine	+	Snell, 1941; Kuhn et al., 1941; McIlwain, 1942, Barnett and Robinson, 1942
Pantoyltauramide	+	McIlwain, 1942; Barnett and Robinson, 1942
α -Methylpantothenic acid	+	Pollack, 1943
Panthydrazide	+	Madinaveitia et al., 1945
Pantoyldialkylaminoalkylamines	+	Madinaveitia et al., 1945
Pantoyl- β -aminoethylphenylsulfones and substituted compounds of this type	+	Madinaveitia et al., 1945; Seneor et al., 1947
N-Pantoyl- β -aminoethylthiol	+	Barnett, 1944
Bis-(pantoyl- β -aminoethyl) monosulfide, disulfide, sulfoxide, sulfone	+	Barnett, 1944
N-Pantoylethanolamine, N-pantoyl-n-propylamine	+	Snell and Shive, 1945
N-Pantoylglycine	+	Snell and Shive, 1945; Nielsen and Roholt, 1945
N-Pantoyl- β -aminoisobutyric	+	Snell and Shive, 1945
N-Pantoyl-4-amino-2-butanol	+	Snell and Shive, 1945
N-Pantoyl- β -aminobutyric	+	Snell and Shive, 1945; Nielsen and Roholt, 1945
N-Pantoylisoserine	+	Snell and Shive, 1945; Nielsen and Roholt, 1945
N-Pantoylalkylamines	+	Shive and Snell, 1945
N-Pantoyl- β -methoxyethylamine	+	Shive and Snell, 1945
N-Pantoyl- β -phenylethylamine	+	Shive and Snell, 1945
Pantothenonitrile	+	Shive and Snell, 1945
Pantothenylamine	+	Shive and Snell, 1945
Pantoyl- α -aminovaleric acid	+	Nielsen and Roholt, 1945
Pantoyl- ϵ -aminocaproic acid	+	Nielsen and Roholt, 1945
Pantoylvaline	—	Nielsen and Roholt, 1945
Pantoylserine	—	Nielsen and Roholt, 1945
Pantoylasparagine	—	Nielsen and Roholt, 1945
Pantoylphenylalanine	—	Nielsen and Roholt, 1945
Pantoylsulfanilamide	—	Nielsen and Roholt, 1945
Pantoyl-p-aminobenzoic acid	—	Nielsen and Roholt, 1945
Phenyl pantothenone	+	Woolley, 1944, 1945
Pantothenyl alcohol	+	Snell and Shive, 1945
D-[2-(Pantoylamino)ethylsulfonyl 4-nitroanilide	+	Winterbottom et al., 1947; Cohen et al., 1949

Table I—(Continued)

Compound	Activity	Reference
(+)-p-Chlorophenylpantothenone . . .	+	Lutz et al., 1947
(+)-p-Tolylpantothenone, also corresponding alcohol	+	" "
dl-N-Pantoyl-β-phenylethylamide	+	" "
Pantoyl-β-aminoethylphenylsulfide . .	+	Senear et al., 1947; Singher et al., 1948
Pantoyl-β-aminoethylphenylsulfoxide	+	" "
Pantoyl-β-aminoethyl-p-chlorophenyl sulfide & sulfone	+	" "
d-N ² -(Pantoyltauryl)-2- aminopyridine	+	Mead et al., 1946; Winterbottom et al., 1947
d-N ² -(Pantoyltauryl)-2- aminopyrimidine	+	" "
d-N ² -(Pantoyltauryl)-2- aminothiazole	+	" "
d-Pantoyltaurylaminocyclohexane . . .	+	" "
d-Pantoyltaurylaminooctadecane	+	" "
Pantoyltauryl-9-aminoanthracene	+	" "
d-[2-(Pantoylamino)-ethylsulfono]- 4-aminoanilide	+	Winterbottom et al., 1947
d-2-[2-(Pantoylamino)-ethyl- sulfonamido]-5-chloropyridine	+	" "
d-2-[2-(Pantoylamino)-ethyl- sulfonamido]-5-bromopyridine	+	" "
d-2-[2-(Pantoylamino)-ethyl- sulfonamido]-5-chloropyrimidine . .	+	" "
d-2-[2-(Pantoylamino)-ethyl- sulfonamido]-5-bromopyrimidine . .	+	" "
d-2-[2-(Pantoylamino)-ethyl- sulfonamido]-quinoline	+	" "
d-[2-(Pantoylamino)-ethyl- sulfonamido]-benzene	+	" "
d-[2-(Pantoylamino)-ethyl- sulfonamido]-4-chlorobenzene	+	" "
d-[2-(Pantoylamino)-ethylsulfon- amido]-3,5-dibromobenzene	+	" "
d-[2-(Pantoylamino)-ethyl- sulfonamido]-4-toluene	+	" "
d-[2-(Pantoylamino)-ethyl- sulfonamido]-4-methoxybenzene . .	+	" "
d-[2-(Pantoylamino)-ethyl- sulfonamido]-4-carboxybenzene . . .	+	" "
d-2-[2-(Pantoylamino)-ethyl- sulfonamido]-naphthalene .	+	" "

taurine and tauramide analogues and obtained similar biological results.

Results obtained by McIlwain (1942) with *Proteus morganii* indicate that the same enzyme systems are probably not affected in all cases. *Proteus morganii* required practically the same amount of pantothenate as the sensitive streptococci but it required 100 times as much pantoyltaurine to inhibit *morganii* as to inhibit the streptococci. All sensitive organisms considered by McIlwain needed added pantothenate for optimal growth. Gradations from complete sensitivity to complete resistance occurred in one and the same microorganism, namely *Corynebacterium diphtheriae*. The amount of pantothenate produced by resistant bacteria (concentration in situ) was insufficient to antagonize the effects of the taurine analogue on a sensitive organism (pantothenate added). This would imply that the pantothenate produced in situ is more effective as a reversing agent than added pantothenate.

McIlwain (1942) emphasizes the point that, in contrast to the sulfanilic acid-sulfanilamide interrelationship, pantoyltaurine is more effective than its amide. The inhibitions produced by the two are of a single type in contradistinction to that of pyridine-3-sulfonic acid and its amide where the action is directed specifically against nicotinic acid or against nicotinamide.

As an example of quantitative relations, concentrations of pantothenate 10^{-7} molar were inhibited by pantoyltaurine at levels of 2×10^{-5} molar where streptococci were the test organisms (McIlwain 1942a).

McIlwain (1944), extending his studies of the basic mechanisms involved in the action of pantoyltaurine, came to the conclusion that pantothenate is important to microorganisms because the products to which it normally leads are necessary in adequate concentrations for normal growth. He reached this hypothesis following a study of the growth of streptococcal cultures as reflected in their gaseous metabolism. Apparently pantoyltaurine acts by lowering the rate of formation of these products of pantothenic acid metabolism to such an extent that the growth rate is reduced. Further work along these lines led McIlwain and Hughes (1944) to observe a reaction of hemolytic streptococci, involving pantothenate usage, inhibited by pantoyltaurine, and associated with carbohydrate metabolism. Pantothenate disappeared from streptococcal cultures due to a reaction for which the organisms were necessary but which was independent of their growth and viability. This metabolism did not depend on oxygen supply but was associated with the presence of glucose and amino acids. Inhibition of glycolysis occurred in direct correlation with inhibition of pantothenate metabolism. Of all agents tested, pantoyltaurine was the only one which inhibited pantothenate metabolism without a corresponding inhibition of glycolysis.

Similar observations (McIlwain, 1944a) led to the conclusion that inasmuch as the concentrations of pantoyltaurine inhibiting growth or metabolism varied independently of the rate at which organisms inactivated

pantothenate, or performed the glycolysis which was associated with pantothenate catabolism whether pantoyltaurine resistant or not, there was no basis for considering the number of receptor sites greater in resistant than in sensitive bacteria. The receptors however appear to differ qualitatively.

McIlwain and Hughes (1945) demonstrated a correlation between the capacities of pantoyltaurine to inhibit growth of bacteria and to prevent the inactivation of pantothenic acid by these bacteria during glycolysis. The failure of pantoyltaurine concentrations for growth inhibition to correlate with pantothenate inactivation by different organisms combined with the existence of such correlation for any given organism is additional justification for the concept of qualitative receptor difference. These workers studied 14 compounds structurally related to pantothenate and found seven which inhibited the growth of hemolytic streptococci and the inactivation of pantothenate, the two activities being directly correlated. Five compounds affected neither process; two compounds which inhibited growth and which were not antagonized by pantothenate did not affect pantothenate inactivation. In the series studied by these investigators, the compounds which inhibited pantothenate inactivation were all amides of pantoic acid. A latent period for activity of pantoyltaurine against β -hemolytic streptococci is observed (McIlwain, 1945). Low concentrations of pantoyltaurine will prevent commencement of the bacterial growth, but if the growth has started, the addition of even relatively high concentrations will not affect its progress until after a latent period of an hour or more. The reverse is also true: the effect of pantothenic acid in counteracting the antibacterial action of pantoyltaurine is a delayed one. The inhibition of pantothenate inactivation by pantoyltaurine is an immediate reaction. McIlwain (1945) indicates that the latent period for activity of pantoyltaurine on growth in an already growing culture is due to the presence in the cells of the preformed metabolic products of pantothenic acid which are the true growth factors. This work by McIlwain implies the existence of molecules of the type of coenzyme A (Lipmann et al., 1947) and foreshadows the failure of metabolite analogues of pantothenic acid to affect the action of preformed coenzyme A (Novelli and Lipmann, 1948; Martin et al., 1950).

Further work on the possible displacement of p-aminobenzoic acid and of pantothenic acid from microbial cells, confirmed his earlier findings and caused McIlwain (1944a, 1945a) to state that no displacement occurred and to conclude that these substances act as bacteriostatic agents by preventing the binding in functioning form of pantothenate and p-aminobenzoate by bacteria. This does not in any way modify the significance of the use of the term, displacer, because it does not imply displacement of bound metabolite any more than it implies displacement by competition for a reaction site. An agent that acts by preventing the binding of a chemotherapeutic agent on a cell structure is equally a displacing agent. In this observa-

tion of Mellwain (1945a) we have additional material which can be integrated into the coenzyme A concept.

In 1943, Pollack synthesized a new and interesting analogue of pantothenic acid . . . α -methylpantothenic acid. This compound had a repressive action for the growth effect of the vitamin on *Lactobacillus casei* and the action could be overcome by the addition of more pantothenic acid. Under certain conditions, this α -methyl homologue acted as a growth stimulant.

Several other groups of analogues have been reported with β -alanine moiety modifications. Thus, Madinaveitia et al. (1945) prepared pantamides related to pantothenic acid, including panthydrazide, the amides of dialkylaminoalkylamines, and substituted aminoethylphenyl sulfones. Several exhibited marked antagonism toward pantothenic acid with respect to growth of *Lactobacillus casei* and *Streptococcus hemolyticus*. Of those examined for therapeutic activity in rats infected with streptococci, only one, di- β -(N-pantoylaminoethyl)-p-aminophenyl sulfone, showed any activity and that but slight.

Among other analogues of pantothenic acid of this type are the sulfur-containing substances (Barnett, 1944) which include N-pantoyl- β -aminoethylthiol, bis(pantoyl- β -aminoethyl) monosulfide, disulfide, sulfoxide, and sulfone. None was more active than pantoyltaurine against *Lactobacillus arabinosus*, but the disulfide and the thiol had an almost equal inhibitory action, which was reversed by pantothenic acid. In vivo tests showed these compounds were not superior to pantoyltaurine. Three aryl substituted analogues of pantoyltaurine were prepared (Barnett et al., 1944) but none showed activity either in vivo or in vitro.

Snell and Shive (1945) prepared pantothenyl alcohol, N-pantoylethanolamine, N-pantoylglycine, N-pantoyl-n-propylamine, N-pantoylallylamine, and N-pantoylethylamine. The susceptibility of various microorganisms to growth inhibition by pantothenyl alcohol and pantoyltaurine differed markedly; an organism may be sensitive to one and resistant to the other. The sensitivity of *Leuconostoc mesenteroides* for the compounds varied as follows: pantothenyl alcohol > pantoylethanolamine > pantoylallylamine > pantoylpropylamine > pantoylethylamine > pantoylglycine.

N-Pantoyl-4-amino-2-butanol, sodium N-pantoyl- β -aminobutyrate, sodium N-pantoyl- β -aminoisobutyrate and N-pantoylisoserine were prepared and tested by these investigators (Shive and Snell, 1945d and Snell and Shive, 1945). All of the compounds inhibited the growth of microorganisms which require pantothenic acid. The inhibition was competitive in nature. N-Pantoyl- β -aminobutyric acid (β -methylpantothenic acid) was the most versatile of the compounds tested.

Extending their investigations, Shive and Snell (1945) synthesized dl-N-pantoylalkylamines, dl-N-pantoyl- β -methoxyethylamine, dl-N-pantoyl- β -

phenylethylamine, dl-pantothenonitrile, dl-pantothenylamine. All inhibited the growth of pantothenic acid requiring microorganisms, the effect in each case being counteracted by pantothenic acid.

The quest for highly effective pantothenic acid displacers caused Nielsen et al. (1944) to extend their studies of analogues of β -alanine and to include those of pantothenic acid. They reported no growth inhibiting action of methyl pantothenic acid against *Streptobacterium plantarum*. In 1945, their work (Nielsen and Roholt, 1945) was extended to a series of pantoyl amino acids including pantoyl- β -aminobutyric acid, pantoyl- δ -aminovaleric acid, pantoyl- ϵ -aminocaproic acid, pantoylglycine, and pantoylisoserine. These were all active. Pantoylvaline, pantoylserine, pantoylasparagine, pantoylphenylalanine, and pantoylsulfanilamide were inactive. Pantoyl-p-aminobenzoic acid was an inhibitor of bacterial growth but its action was not that of an antivitamin.

Woolley (1944, 1945) states that phenyl pantothenone (N-(α,γ -dihydroxy- β,β -dimethylbutyryl)-1-aminoethylphenyl-ketone) acted competitively with pantothenic acid. This molecule represents a —CO—R replacement of a carboxyl radical. Methyl pantothenone inhibited bacterial growth but was not counteracted by pantothenic acid. Aminoethylphenylketone was ineffective.

Extending his observation with phenylpantothenone, Woolley (1946) found that the inhibitory action of this molecule could be reversed by certain amino acids in all species examined. This was in contrast to his finding (1944, 1945) that the antimicrobial action of phenylpantothenone was reversed by pantothenic acid only in those species which were stimulated in growth by this vitamin. For *Saccharomyces cerevisiae*, histidine was the most active amino acid. Glutamic acid, proline, aspartic acid, asparagine, glycine, serine, threonine, alanine, and lysine all displayed some activity. The other amino acids were inactive. Woolley emphasizes the fact that this finding is not unique in the study of the relationships of metabolites and their competing analogues. It is seen in the p-aminobenzoic acid-sulfanilamide system, wherein methionine and adenine are capable of displacing the antibacterial action of sulfonamides. This polyphasic displacement specificity is a result of the complexity and multiplicity of metabolic channels.

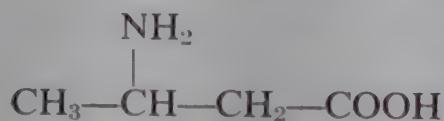
An interesting relationship is to be noted between this work and that of Ravel and Shive (1946) who reported the capacity of cysteic acid to prevent the synthesis of pantothenic acid in *Escherichia coli* by blocking the enzymatic decarboxylation of aspartic acid to β -alanine, an observation which demonstrates further the extreme complexity of the application of the metabolite displacer concept to chemotherapy.

It is perhaps true that a molecule as complex as d-(2-(pantoylamino)-ethylsulfono)-4-nitroanilide should be placed in a class by itself; it is

mentioned here, however, because the pantoic moiety is intact. Cohen et al. (1949) synthesized the molecule and report it as an effective antagonist of coenzyme A.

β -Alanine Analogues

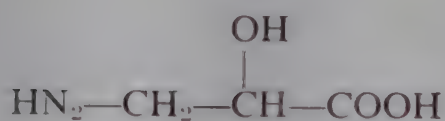
β -Alanine is considered with pantothenic acid rather than in the section on amino acids because its main function is as a precursor of this vitamin. Nielsen and Johansen (1943) found that a 5000 fold concentration of β -aminobutyric acid (Fig. 1) nullified the growth promoting action of β -alanine on yeast. They also tested isoserine (α -oxy- β -aminopropionic acid)



β -Aminobutyric acid

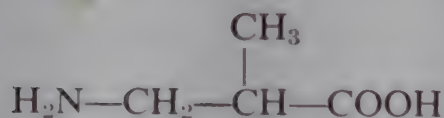


β -Alanine



Isoserine

(α -hydroxy- β -aminopropionic acid)



α -Methyl- β -alanine

FIG. 1. β -Alanine and displacers.

and β -methylaminopropionic acid against β -alanine. None of the compounds were growth factors in the absence of β -alanine and pantothenic acid. β -Aminobutyric acid and isoserine definitely inhibited the growth promoting action of β -alanine, the inhibitory effect of the β -aminobutyric acid being the strongest. None of these chemicals showed any action against pantothenic acid. The conclusion was drawn that the inhibiting chemical was formed into a compound resembling pantothenic acid but with no growth promoting value. This was proposed as a new type of displacement, in which the compound actually functioning would be the metabolic analogue of pantothenic acid, the effect being initiated by an enzymatic mechanism which incorporated β -aminobutyric acid into the molecule in place of β -alanine.

In 1944, this same group (Nielsen et al., 1944) reported the counter-

action of the growth promoting effect of β -alanine in yeast by another analogue, phenyl- β -alanine. They found that most α -amino acids antagonized β -alanine. Cysteine and methionine caused a general inhibitory action. Along these same lines, antagonism with β -alanine has been demonstrated by Wright and Skeggs (1946) to form the basis of the bacteriostatic action of sodium propionate on *Escherichia coli*. None of the other amino acids studied possessed this characteristic.

Another approach to the problem deals with the prevention of the formation of pantothenic acid. This would reduce itself to a study of the capacity of pantoic acid analogues to inhibit the formation of the vitamin. Cheldelin and Schink (1947) used *Acetobacter*, which utilizes pantoic acid as readily as the intact vitamin, as a test microorganism. β,β -Dimethyl- γ -hydroxybutyric acid; α -hydroxy- β,β -dimethylbutyric acid; β -methyl- β , γ -dihydroxybutyric acid all competitively inhibited the coupling of β -alanine to pantoic acid in *Acetobacter suboxydans*. Later, this same laboratory (King et al., 1948) recorded the reversal by pantothenic acid of the inhibition of growth of *Escherichia coli* through 2-chloro-4-aminobenzoic acid. These findings were confirmed by Hartelius (1943).

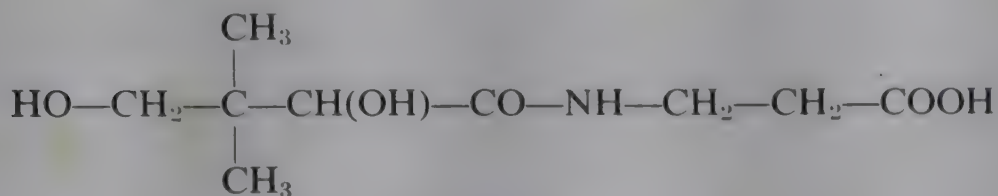
Pollack (1943) synthesized α -methyl- β -alanine (Fig. 1) and found that it could replace β -alanine as a growth stimulant for Gebruder Mayer yeast, though it was only about 0.0006 as potent. At higher levels this compound showed a slight repressing action on the growth effect of β -alanine. One rather unexpected development of this study was the finding that α -methyl- β -alanine was antagonistic where α -methyl pantothenic acid was not; this in spite of the probability that the simpler molecule would be combined into the more complex.

This observation largely discredits the proposals of Nielsen and Johansen (1943) and supports the concept that metabolite analogues of β -alanine act by prevention of the formation of pantothenic acid.

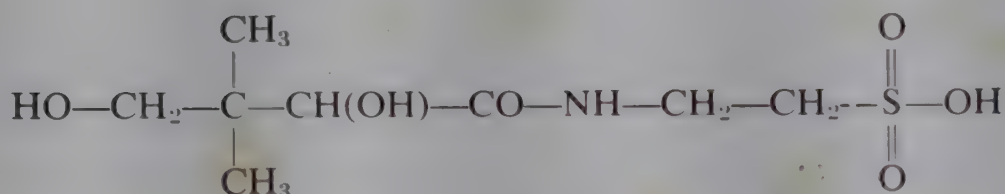
Analogues with Pantoic Acid Moiety Modification

Mitchell et al. (1940) first observed an effect on bacterial growth produced by hydroxypantothenic acid, which Knight (1945) later suggested represented a type of inhibition by blockage of the metabolite from its active enzyme site. Hydroxypantothenic acid is therefore not only the first metabolite antagonist of pantothenic acid ever reported but also the first example of an antagonist in which the pantoic acid moiety has been modified.

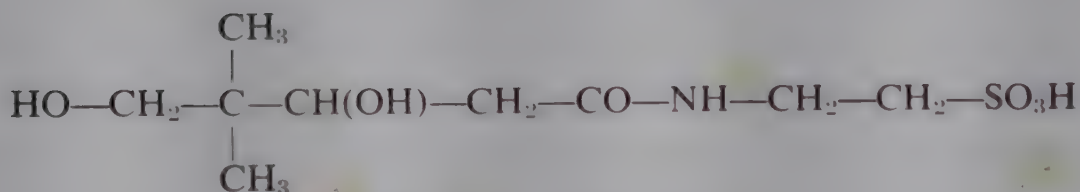
γ -Hydroxy-n-butyryl- β -alanide (Fig. 2) (McIlwain, 1942) was the second analogue of this type to be synthesized. Its action was complementary to that of certain other analogues, such as pantoyltauramide, in that the compounds were effective against different groups of organisms. This analogue (γ -hydroxy-n-butyryl- β -alanide) was active against a strain of *Corynebacterium diphtheriae* which did not require added pantothenate,



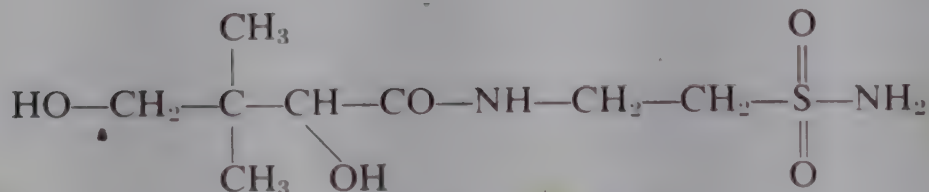
Pantothenic acid
(α,γ -dihydroxy- β,β -dimethylbutyryl- β' -alanide)



Pantoyltaurine
(α,γ -dihydroxy- β,β -dimethylbutyryl-*taurine*)



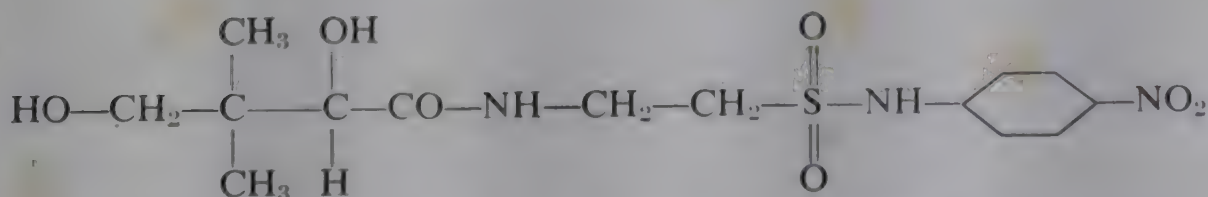
Homopantoyltaurine



Pantoyltauramide



γ -Hydroxy-*n*-butyryl- β -alanide



D-[2-(Pantoylamino)-ethylsulfonyl]-4-nitroanilide
D-(pantoyltauryl)-4-nitroanilide

FIG. 2. Pantothenic acid and displacers.

and was ineffective against those which did. This fundamental difference was interpreted by McIlwain (1942) as indicating that the action of analogues such as pantoyltaurine is one of primary displacement of pantothenate, whereas the other analogue (γ -hydroxy-n-butyryl- β -alanide) inhibited the formation of pantothenate by virtue of its similarity to the product of an enzyme action.

Clostridium septicum has been reported as incapable of synthesizing the pantoyl moiety of the pantothenate molecule (Ryan et al., 1945) while it can synthesize β -alanine and is able to couple it with the pantoyl moiety if this is present in the medium. This organism should be susceptible to γ -hydroxy-n-butyryl- β -alanide and resistant to the action of pantoyltaurine. Failure of pantoyltaurine to inhibit the growth of the gonococcus has been reported (Kimmig, 1943) and a similar situation may obtain for this micro-organism.

In view of the foregoing, the observation of McIlwain (1942) to the effect that a tryptic digest of casein inhibited the action of the γ -hydroxy-n-butyryl- β -alanide on *Escherichia coli* suggests a specific structural similarity to some amino acid resembling the γ -hydroxy-n-butyryl portion of the molecule, which would be serine or glutamic acid. It will be recalled that phenylpantothenone, which is a representative of the series with β -alanine moiety modification, is also counteracted by amino acids (Woolley, 1946).

Table II

METABOLITE ANALOGUES OF PANTOTHENIC ACID WITH PANTOIC ACID MOIETY MODIFICATIONS

Compound	Activity	Reference
(+)- β -Hydroxy- γ,γ -dimethylvaleryl- β -alanide	+	Barnett and Robinson, 1942
N-(α -Hydroxy- β,β -dimethylbutyro)- β -alanide	+	Cheldelin and Schink, 1947
N-(β -Methyl- β,γ -dihydroxybutyro)- β -alanide	+	Cheldelin and Schink, 1947
N-(γ -Hydroxy- β,β -dimethylbutyro)- β -alanide	+	Cheldelin and Schink, 1947
N-(α,α -Dimethyl- β -hydroxypropionyl)- β -alanide	—	Lipton and Strong, 1949
B'-(α -Amino- β,β -dimethyl- γ -hydroxy-butyrylamino) propionic acid	—	Holly et al., 1948
N-(α,γ -Dihydroxy- β,β -dimethylvaleryl)- β -alanide	+	Drell and Dunn, 1946
ω -methylpantothenic acid		
γ -Hydroxy-n-butyryl- β -alanide	+	McIlwain, 1942
Hydroxypantothenic acid	+	Mitchell et al., 1940
Salicyloyl- β -alanide	+	Moss et al., 1948
Mandelyl- β -alanide	+	Moss et al., 1948
Homopantoyl- β -alanide	—	McIlwain and Hughes, 1945

Drell and Dunn (1946) synthesized N-(α,γ -dihydroxy- β,β -dimethylvaleryl)- β -alanide and demonstrated its capacity to displace pantothenic acid. The compound was designated as ω -methylpantothenic acid.

Another group of molecules in which metabolite displacers for pantothenic acid were produced by pantoic acid moiety modifications was presented by Moss et al. (1948): included were salicyloyl- β -alanide and mandelyl- β -alanide.

It is of interest to note that a subsequent investigation (Martin et al., 1950) of the capacity of a series of pantothenic acid displacers to inhibit the action of choline acetylase disclosed that only salicyloyl- β -alanide possessed this function and that it was a nonspecific action, being incapable of reversal by pantothenic acid.

These findings tend to invalidate the position taken by Cheldelin and Schink (1947) that analogues of pantothenic acid are generally good growth inhibitors if the β -alanine structure is modified, and that if the pantoic acid moiety is modified, the compounds are slightly stimulatory or inert. The new analogues of pantothenic acid prepared in their study were the corresponding β -alanides or taurine compounds of β,β -dimethyl- γ -hydroxybutyric acid, α -hydroxy- β,β -dimethylbutyric acid and β -methyl- β,γ -dihydroxybutyric acid.

Another analogue of pantothenic acid with a pantoic modification was prepared by Holly et al. (1948) and designated as an amino analogue . . . β' (α -amino- β,β -dimethyl- γ -hydroxybutyrylamino)-propionic acid. The molecule had no antagonistic action to the growth effect of pantothenic acid. Lipton and Strong (1949) prepared in crude form the keto analogue of pantothenic acid. It had no vitamin activity but apparently was not tested as a displacer. Another compound of their series, N-(α,α -dimethyl- β -hydroxypropionyl)- β -alanide was inactive as displacer and as vitamin.

Analogues with Both Pantoic Acid and β -Alanine Moiety Modification

Homopantoyltaurine synthesized by McIlwain (1942) is an example of an effective metabolite antagonist in which both the pantoic acid and the β -alanine moiety of the pantothenic acid molecule have been changed. While homopantoyltaurine possessed a lower degree of activity than either pantoyltaurine or pantoyltauramide, it was nevertheless a true displacing agent.

Another example was supplied by Barnett and Robinson (1942) who prepared the taurine compound of β -hydroxy- γ,γ -dimethylvalerolactone. Tested on streptococci, the molecule was highly inhibitory and could be reversed by pantothenic acid.

In still another series of analogues showing inhibitory activity not reversed by pantothenic acid, it was observed that the alpha hydroxy group of pantothenic acid was absent.

Table III

METABOLITE ANALOGUES OF PANTOTHENIC ACID WITH MODIFICATION IN BOTH β -ALANINE AND PANTOIC ACID MOIETY

Compound	Activity	Reference
N-(α -Hydroxy- β,β -dimethylbutyro)-taurine	—	Cheldelin and Schink, 1947
N-(α,γ -Dihydroxy- β -methyl- β -ethylbutyro) taurine	+	Wieland and Möller, 1948
Homopantoyltaurine	+	McIlwain, 1942
N-(β -Hydroxy- γ,γ -dimethylvaleryl)-taurine	+	Barnett and Robinson, 1942
N-(α,γ -Dihydroxy- β,β -dimethylvaleryl)-taurine	+	Drell and Dunn, 1948
N-(α,γ -Dihydroxy- β,β -dimethylvaleryl) leucine	+	Drell and Dunn, 1948
γ -Hydroxy-n-butyryltaurine	+	Moss et al., 1948
N'-Diacetylpantoylsulfanilamide	+	Moss et al., 1948

A few years after their report on ω -methylpantothenic acid, Drell and Dunn (1948) reported the corresponding taurine and leucine compounds of α,γ -dihydroxy- β,β -dimethylvaleric acid. The taurine compound was less active than the β -alanine molecule, and the leucine analogue least active. A comparison of the activity of ω -methylpantothenic acid, pantoyltaurine, and ω -methylpantoyltaurine disclosed the fact that reaction sites blocked by ω -methylpantothenic acid and pantoyltaurine differed and that the hybrid molecule ω -methylpantoyltaurine occupied an intermediate position seeming to possess the capacity to inhibit both types of reaction position. This work discloses again the probability that the use of pantothenic acid displacer types in conjunction should comprise a more effective chemotherapy or pharmacological reactor.

Wieland and Möller (1948) synthesized still another pantothenic acid analogue with displacer characteristics in which both the pantoic acid and the β -alanine moiety of the structure were changed. The molecule was N-(α,γ -dihydroxy- β -methyl- β -ethylbutyro) taurine.

Compounds Reported to Act by Virtue of Interference with Pantothenic Acid Metabolism

Ivanovics (1942) reported a relationship between the pantothenic acid molecule and the action of sodium salicylate as a bacterial growth inhibitor. The inhibitory action of salicylate was counteracted by the addition of pantothenic acid, α,γ -dihydroxy- β,β -dimethylbutyric acid, acetylsalicylic acid and salicylic acid phenyl ester, but not by alanine, thiosalicylic, or salicylamide. The suggestion was made that the action of salicylates in low concentrations is due to specific effects of salicylate on the synthesis of pantothenic acid. These observations lead to speculation that the analgesic

action of salicylates may be due to the inhibition of pantothenic acid formation or utilization and this opens a field for the testing of pantothenic acid displacers. Confirmation and extension of this work has appeared (Ivanovics, 1942a; Nitti and Faquet, 1944). As suggested by Ivanovics (1942), antagonism of the salicylate inhibition of the growth of *Escherichia coli* is a characteristic of pantoic acid and to a lesser degree of pantolactone (Stansly and Schlosser, 1945; Stansly and Alverson, 1946).

A second aromatic acid, mandelic (Perould and Greib, 1944) functions by virtue of its interference in bacterial metabolic systems involving pantothenic acid. The action of mandelic acid on *Escherichia coli* was found to be counteracted by 0.000,001 M pantothenic acid.

McIlwain (1943) attempted to utilize the concept of a synergism by a combination of a pantothenic acid displacer with a compound preventing the formation of this vitamin, when he demonstrated the action of salicylate and pantoyltaurine on *Proteus morganii*; however, the salicylic effect was apparently due to the protein denaturing properties of salicylic acid (Ivanovics, 1942). A most unusual finding bearing on such interrelationships is that of Stansly and Alverson (1946) who demonstrated an antagonism between pantoyltaurine and salicylate on the growth of *Escherichia coli*. Here is an instance in which two antagonists antagonize each other. The liberation of pantoic acid from pantoyltaurine is offered as the explanation of the observed effect.

A compound which differentiates between fibroblasts and epithelial cells has been described (Medawar et al., 1943) and its action was hypothetically attributed to inhibition of pantothenic acid, an assumption subsequently disproved. The compound through its ability to prevent growth of fibroblasts while permitting growth of epithelial cells should prove valuable in controlling wound healing, preventing excessive fibroblastic activity, preventing overgrowth of connective tissue, and permitting healing without scarring. The compound was α -hexenolactone. Activity was shown in concentrations of 0.006 mg. per cc. α -Valerolactone was not inhibitory.

This work represents an attempted application of the principles of displacement. The fact remains, however, that there are differences in the requirement of various cellular elements of the body for given factors which may make it possible to apply the concept. That there are all grades of quantitative and even qualitative differences in requirements among cells, bacteria, animals, etc. is the very basis on which the practical application of structural displacement stands.

Hauschka (1944) found that while pantothenic acid did not interfere with the activity of hexenolactone, β -alanine, α -alanine, and glutathione did. He suggested that a specific metabolic mechanism involving alanine but not that linked to the biosynthesis of pantothenic acid, was the basis of the differential inhibition and differential tissue destruction by hexenolactone.

An extension of this work (Hauschka et al., 1945) using the planarian *Dugesia tigrina* showed that structural isomers closely related to hexenolactone (ascorbic acid, maleic anhydride, and maleic acid) did not possess the same characteristics. Cysteine but not cystine was an antagonist of this action of hexenolactone. It was suggested by the authors that antibiotics interfere with cellular proliferation mainly through their reactivity with sulfhydryl essential to enzyme functions. Although direct antagonism is not a factor the mechanism is classified by the present author as indirect displacement and the potentialities remain for the application of metabolite analogues in the control of fibroblastic activity during wound healing.

In Vivo Studies

Antibacterial

The opinion has been expressed that although metabolite analogues offer excellent tools for biochemical research they will not prove of value in a practical sense. However, the in vivo work done to date demonstrates the value of these agents in biochemical research and also gives substance to the claim that practical applications await only proper study.

McIlwain and Hawking (1943) investigating the in vivo action of pantooyltaurine, arrived at some conclusions which are fundamental to the entire concept of chemotherapy. They found that pantooyltaurine was rapidly excreted by rats but that by using dosages of 0.25 grams per 100 grams, the pantooyltaurine ratio to pantothenate could be kept above the range necessary for in vitro action. Rats were treated for four days and were then found to be protected from approximately 10,000 LD of a virulent strain of streptococcus and less completely protected against 1,000,000 LD. Mice, whose blood pantothenate is much higher than that of the rat or of man, were not protected. Artificially raising the blood pantothenate of rats also prevented the action of pantooyltaurine. Sulfonamide resistant streptococci and some strains of *Corynebacterium diphtheriae* were found sensitive to pantooyltaurine.

Clearly, the rational approach of chemotherapy lies partially in a consideration of relative concentrations of any given metabolite necessary for life of tissue cells and bacteria. It would be disastrous to use a displacement compound in a human being if the concentration of the displaced compound for the life of the tissue cells exceeded that necessary for the life or reproduction of the microorganisms in question; the result would be the death of the host and not of the invading microorganism. Minimum concentrations for life and growth of dietary factors must be determined for pathogenic bacteria and tissue cells; only with these values in mind can a logical approach be made toward more specific chemotherapeutic principles. Even the diet of the treated patient would have to be watched to

prevent undue variation in metabolite concentration in the blood stream which would introduce an element of unpredictability concerning the action of the chemotherapeutic agent.

Substantiation of the in vivo chemotherapeutic activity of metabolite analogues of pantothenic acid has come from several different laboratories. Di- β -(N-pantoylaminoethyl)-p-aminophenyl sulfone was therapeutically effective against *Streptococcus hemolyticus* infection in rats (Madinaveitia et al., 1945) and the sulfur compounds including N-pantoyl- β -aminoethylthiol, bis(pantoyl- β -aminoethyl) monosulfide, disulfide, sulfoxide, and sulfone were active in vivo, although not more powerfully than pantoyltaurine (Barnett, 1944). The 4-chlorophenyl and the 3,5-dibromophenyl amides of pantoyltaurine were found effective in vivo against *Streptococcus hemolyticus* infection in mice (Winterbottom et al., 1947).

Additional investigation of the in vivo activity of pantothenic acid analogues comes from the work of White et al. (1946). Pantoyltauramido-benzene, naphthalene, pyridine, pyrimidine, and quinoline derivatives were studied. They report that these analogues are highly active in a hemolytic streptococcus infection in mice. Median survival doses of 11 analogues ranged from 4 to 12 milligrams per 20 gram mouse administered as a single oral dose. The antibacterial activity was reversed by pantothenate. The compounds tested were found to be relatively nontoxic.

Drell and Dunn (1948) record the susceptibility of streptococci in vivo to the action of ω -methylpantothenic acid. In ω -methylpantothenic acid, we see an instance of an excellent metabolite displacer for pantothenic acid in vivo in which the pantoic acid moiety is modified.

Production of Pantothenic Acid Deficiency

That many displacement agents can produce vitamin deficiencies has been amply demonstrated, but in the case of pantoyltaurine, controversial findings have been recorded. Snell et al. (1943) reported the production of a pantothenic acid deficiency in mice by feeding pantoyltaurine. The dosage used was 200 mg. per kilogram of body weight. The time for initial appearance of symptoms, e.g., growth effect, was three to four weeks. On the other hand, Woolley and White (1943) found no effect of pantoyltaurine in either mice or hamsters and Unna (1943) reported similar failure using rats. Explanation of these divergent results may lie in species differences associated possibly with the intestinal synthesis of pantothenic acid.

The work of West and his group (1945) assumes increasing importance with the present tendency to interrelate sulfonamides, pantothenic acid, and tryptophan metabolism. They reported that rats receiving a synthetic diet low in casein, containing sulfapyridine, developed a pantothenic acid deficiency syndrome which was corrected by the supplementation of the diet

with calcium pantothenate. This observation ties in with the finding and conclusions of Sevag et al. (1944) to the effect that the inhibition of the synthesis of tryptophan from glucose and amino acids is a factor underlying the action of the sulfonamides, and that pantothenic acid mediates the metabolism of glucose leading to the tryptophan synthesis. Adequate pantothenic acid prevented sulfonamide action. We do not suggest that the limiting factor in the results of West et al. (1945) was necessarily tryptophan, but rather that the same basic mechanism of inhibition of the specific type of glucose metabolism mediated by pantothenic acid might well explain their findings.

Interference of the utilization of pantothenic acid in chicks brought about by aromatic derivatives of pantoyltaurine has been reported (Brackett et al., 1946). Drell and Dunn (1947) tested their compound— ω -methylpantothenic acid—in mice by determining its effect on survival time when added to a pantothenic acid deficient diet. It was an effective displacer having an inhibitor metabolite ratio of approximately 100. Graying of the fur was observed in one of the experimental groups.

Singher et al. (1948) studied extensively a pantothenic acid displacer in the animal. The compound was (—)- α,γ -dihydroxy- β,β -dimethyl-N-(2-(phenylmercapto-)ethyl)-butyramide. The lethal dose was of the order of 5 grams per kilogram. Toxic levels seemed to produce respiratory difficulties. This observation correlates with chronic toxicity studies, which showed that during an 11 day test period the administration of $\frac{3}{16}$ of the lethal dose daily failed to alter significantly the growth rate. This brings into the light the vital importance of the time element in vitamin deficiency states produced by metabolite analogues. It is probable that the susceptibility of cells exposed to absolute or nearly absolute deficiencies will vary in accordance with the length of time during which the cell is exposed. It may be that bacterial cells would succumb much more rapidly than tissue and that the more rapidly metabolizing tissue cells would suffer first. This suggests the possible use of absolute deficiencies, induced by displacers, for treatment of certain malignancies. In the monkey, Singher et al. (1948) gave 3.6 grams of the compound orally over a period of six weeks. During that time there were no digestive or physiological disturbances other than hematological, with a marked decrease in red blood cells and in hemoglobin which upon cessation of administration of the drug returned to normal. The white cells did not vary materially in numbers. Local applications of the displacer to the vaginal tissues did not produce irritation. For example, 20 treatments over a ten day period in rats receiving a total of 20 mg. each produced no changes. Systemically, no consistent effects were noted on blood pressure and heart rate. Respiration became shallow and erratic in the cat following intravenous doses of 10 mg. per kilogram. Smooth muscle was stimulated.

The basis for possible pharmacological application of pantothenic acid displacers came with the work of Lipmann et al. (1947) who announced that the coenzyme for acetylation was a pantothenic acid derivative. This coenzyme had been shown by this same investigator (Lipmann and Kaplan, 1946) to be essential for in vitro acetylation of choline. Defective in vivo acetylation was reported by Riggs and Hegsted (1948) who found that the acetylation of p-aminobenzoic acid was only 50 per cent as great in pantothenic acid deficient animals as in those with normal intakes. The broad role played by pantothenic acid in metabolism makes apparent the significance of the study of pantothenic acid displacers in altering this function. Phenyl pantothenone failed to affect the action of preformed coenzyme A in yeast (Novelli and Lipmann, 1948). Martin et al. (1950) reported that a series of pantothenic acid displacers did not inhibit the action of choline acetylase.

It is felt that failure to displace coenzyme A by means of pantothenic acid displacers simply indicates that to date concentration on structural analogues of pantothenic acid may represent an approach which is too limited in scope. Pantothenic acid incorporated into coenzyme A, which has been demonstrated to contain adenine (Lipmann et al., 1947), doubtless will not be displaced by a structural analogue of pantothenic acid; a structural analogue of the coenzyme appears necessary.

In view of the foregoing it is clear that for applications to pharmacology all studies reported have been of relatively little value. Displacers for pantothenic acid must be designed and synthesized which function against the metabolically active form of the vitamin and not against the vitamin itself. It may be that certain displacers for pantothenic acid will function to prevent the formation of coenzyme A but it is apparent that they will not be effective against preformed coenzyme A.

Antimalarial Studies

During World War II, a preliminary study was made of the antimalarial activity of pantothenic acid displacers. McIlwain and Hawking (1943) first reported pantoyltaurine as being ineffective in *Plasmodium relictum* infections in the canary. Phenylpantothenone was found active against avian malaria by Woolley (1944, 1945, 1946). The major work was outlined in Wiselogle's "Survey of Antimalarial Drugs," 1941-45. Investigations were initiated as a result of the observations of Trager (1943) who found that the addition of calcium pantothenate to a medium containing duck erythrocytes parasitized with *Plasmodium lophurae* lengthened the survival period of the parasites. Pantoyltaurine and other members of the series were tested and found inactive. Pantoyltauramide when administered to chicks intravenously in large doses (2 grams or more per kilogram per day) markedly suppressed the growth of *Plasmodium gallinaceum*. Subse-

quently, phenylpantothenone was tested. It was devoid of activity in ducks infected with *Plasmodium lophurae* but suppressed the parasitemia in chicks infected with *Plasmodium gallinaceum* or *Plasmodium lophurae*. The quinine equivalent of phenylpantothenone was 1.0 (test B-4) or 2.0 (test Q-8). The reason for the difference in accordance with host variation was left unexplained; it did not depend on the difference in blood levels of pantothenic acid in the duck or chick, as these are the same (Trager, 1943). The investigation demonstrated that the efficacy of phenylpantothenone as an antimalarial was due to displacement of pantothenic acid by completely negating its action in chicks given adequate quantities of pantothenic acid.

The conclusion was reached that the pantoic acid moiety of pantothenic acid could not be modified without loss of activity. This, in the present author's opinion, is not a valid position unless it is limited to the series actually tested. The investigators tried various modifications of the β -alanine portion of the molecule and concluded that a variety of changes could be made providing an acyl group (CO, S, SO, SO₂) occupies the same position relative to the molecule as a whole that the carbonyl group of β -alanine occupies in relation to pantothenic acid.

It is felt that the inclusion in this review of Table IV from "The Survey of Antimalarial Drugs" is indicated.

Mead et al. (1946) reported on the synthesis of d-N²-(pantoyltauryl)-2-aminopyridine, d-N²-(pantoyltauryl)-2-aminopyrimidine, N²-(pantoyltauryl)-2-aminothiazole, d-pantoyltaurylaminocyclohexane and the attempted synthesis of pantoyltauryl-9-aminoanthracene. These compounds were tested and the results are incorporated in Table IV.

An extensive investigation of the relationship between pantothenic acid and *Plasmodium gallinaceum* infections in the chicken, and the antimalarial activity of analogues of pantothenic acid was carried out by Brackett and his group at Stamford (Brackett et al., 1946). These investigators used *Plasmodium gallinaceum* infections in the chicken, because it was known that well-controlled disease states could be produced and the pantothenic acid nutrition of the chicken had been thoroughly studied. Pantothenic acid deficiency states produced by dietary means strikingly reduced the trophozoite-induced infections. There was also a reduction in the number of merozoites produced by segmenters in the deficient hosts. In direct contrast, the sporozoite-induced infections were not markedly affected, if at all.

It is interesting to note that in these experiments the intensity of infections can be directly correlated with blood levels of pantothenic acid. Variation in level from 0.155 γ per ml. to 0.24 γ per ml. of pantothenic acid in the blood altered the course of the malarial infections. This demonstrates clearly the importance of differential requirement of host and parasite. It will be this differential requirement which will in all cases of the application

Table IV
PANTOTHENIC ACID ANALOGUES AS ANTIMALARIALS

Survey Number	Configuration*	$ \begin{array}{c} \text{CH}_3 \quad \text{OH} \\ \quad \\ \text{HO} - \text{CH}_2 - \text{C} - \text{CH} - \text{C} - \text{NR}_2 \\ \quad \\ \text{CH}_3 \quad \text{O} \\ \text{--- NR}_2 \end{array} $	Test and Antimalarial Activity
14,614	D	—NHCH ₂ CH ₂ CH ₃	O—1† Q < 0.06
14,254	DL	—NHCH ₂ CH ₂ C ₆ H ₅	B—4 Q 0.03
15,032	D	—NHCH ₂ CH ₂ CH(OH)—p— —C ₆ H ₄ CH ₃	A—1 Q 1.0
5,210	D	—NHCH ₂ CH ₂ COOH	A—1 Q < 0.015
3,292	D	—NHCH(COOC ₂ H ₅)CH ₂ COOC ₂ H ₅	Q—1 Q < 0.015
12,610	D	—NHCH ₂ CH ₂ COC ₆ H ₅	B—4 Q 0.8
13,767	L	—NHCH ₂ CH ₂ COC ₆ H ₅	B—4 Q < 0.15
14,688	D	—NHCH ₂ CH ₂ CO—p—C ₆ H ₄ CH ₃	B—4 Q 1.0
13,776	D	—NHCH ₂ CH ₂ CO—p—C ₆ H ₄ Cl	B—4 Q 3
9,973	D	—NHCH ₂ CH ₂ COC ₆ H ₂ (2—CH ₃)— —(4,5—Cl ₂)	B—1 Q 1.0
327	D	—NHCH ₂ CH ₂ SO ₃ H	Q—1 Q < 0.006
13,592	D	—NHCH ₂ CH ₂ SC ₆ H ₅	B—4 Q 0.8
14,799	D	—NHCH ₂ CH ₂ S—p—C ₆ H ₄ Cl	A—1 Q 1.0
13,593	D	—NHCH ₂ CH ₂ SOC ₆ H ₅	B—4 Q 1.5
13,594	D	—NHCH ₂ CH ₂ SO ₂ C ₆ H ₅	B—4 Q 1.0
3,279	D	—NHCH ₂ CH ₂ SO ₂ NH ₂	Q—1 Q 0.03
7,469	D	—NHCH ₂ CH ₂ SO ₂ NHCH ₃	Q—1 Q 0.004
7,468	D	—NHCH ₂ CH ₂ SO ₂ N(CH ₃) ₂	Q—1 Q 0.002
3,282	D	—NHCH ₂ CH ₂ SO ₂ NHC ₁₈ H ₃₇	Q—4 Q < 0.08
3,633	D	—NHCH(CH ₂ COOH)—p—C ₆ H ₄ NH ₂	Q—1 Q < 0.0015
14,621	D	—NHCH ₂ CH ₂ SO ₂ NHC ₆ H ₅	O—1† Q 10
14,620	D	—NHCH ₂ CH ₂ SO ₂ NH—p— —C ₆ H ₄ CH ₃	O—1 Q 4
14,797	D	—NHCH ₂ CH ₂ SO ₂ N(C ₂ H ₅)C ₆ H ₅	O—1† Q 1.0
14,622	D	—NHCH ₂ CH ₂ SO ₂ NH—p—C ₆ H ₄ Cl	O—1† Q 10
14,625	D	—NHCH ₂ CH ₂ SO ₂ NH—3,5—C ₆ H ₃ Br ₂	O—1† Q 0.6
14,618	D	—NHCH ₂ CH ₂ SO ₂ NH—2—naphthyl	O—1† Q 0.6
5,923	D	—NHCH ₂ CH ₂ SO ₂ NH—9—anthryl	Q—4 Q < 0.08
9,667	Not given	—NHCH ₂ CH ₂ SO ₂ NH—2—thiazolyl	Q—1 Q < 0.006
3,280	D	—NHCH ₂ CH ₂ SO ₂ NH—2—pyridyl	O—1† Q 0.3
14,617	D	—NHCH ₂ CH ₂ SO ₂ NH(5—chloro— —2—pyridyl)	O—1† Q 1.0
14,616	D	—NHCH ₂ CH ₂ SO ₂ NH(5—bromo—2— —pyridyl)	O—1† Q 1.0
14,619	D	—NHCH ₂ CH ₂ SO ₂ NH—2—quinolyl	O—1 Q 0.6
7,293	D	—NHCH ₂ CH ₂ SO ₂ NH—2—pyrimidyl	Q—1 Q < 0.03
14,798	D	—NHCH ₂ CH ₂ SO ₂ NH(5—chloro—2— —pyrimidyl)	O—1† Q 0.6

* Configuration is given relative to D-pantothenic acid, which is considered for the purpose of this table to have the D configuration.

† Drug administered in diet 2 days before and 7 days after infection. (From Wiselogle, "A Survey of Antimalarial Drugs," Table 29.) (Courtesy, Edwards Brothers, Inc., Ann Arbor, Mich.)

of the displacement concept to chemotherapy determine the practical applicability. Fifteen pantothenic acid displacers were tested and all exhibited antimalarial activity to a degree. The effectiveness paralleled precisely the dietary-induced pantothenic acid deficiency results. In sporozoite infected chickens there was no activity whatever. The most active compound studied was pantoyltauramide-4-chlorobenzene which was four times as active as quinine in a standard four day trophozoite-induced infection and 16 times as active in blood-induced infections of seven day duration. In these experiments, eight times as much pantothenic acid as displacer was required to nullify the action of the displacers on the malarial infection. The authors state "The wide range between the toxic and therapeutically effective doses in this particular host-parasite relationship is emphasized, since the compounds would undoubtedly be safe drugs in any similar relationship." A striking increase in the immature erythrocytes in the circulating blood was produced by pantoyltauramido-4-chlorobenzene.

A possible explanation of the immunity of the exoerythrocytic parasite to pantothenic acid deficiency and to pantothenic acid displacers offered by these workers is that it is due to the much higher concentrations of pantothenic acid in tissues as contrasted to blood. Snell et al. (1940) noted in various tissues of deficient chickens a pantothenate concentration 100 times that in the blood. The ineffectiveness of pantothenic acid analogues against coccidiosis in the fowl is mentioned by Brackett et al. (1946).

The series of compounds used in the studies of Brackett et al. (1946) had been prepared by Winterbottom and his group (1947). The entire series of amides of pantoyltaurine showed both antibacterial and antiplasmodial activity. The compounds were synthesized by condensing taurine with phthalic anhydride to form the potassium salt of 2-phthalimidoethanesulfonic acid, which was then converted to 2-phthalimidoethanesulfonyl chloride. This latter compound was reacted with the proper heterocyclic or isocyclic amine which resulted in the formation of the corresponding 2-phthalimidoethane sulfonamides. Cleavage of the phthaloyl derivative was made to the corresponding 2-ethanesulfonamide, which was then condensed with pantolactone. In vivo activity was demonstrated against *Streptococcus hemolyticus*, strain C 203, using the 4-chlorophenyl and the 3,5-dibromophenyl amides of pantoyltaurine.

The pantoyl derivatives of β -aminoethyl phenyl sulfide, β -aminoethyl phenyl sulfone, β -aminoethyl phenyl sulfoxide, β -aminoethyl-p-chlorophenyl sulfide and β -aminoethyl-p-chlorophenyl sulfone were synthesized by Senear et al. (1947). All were found to possess antimalarial activity against blood-induced infections of *Plasmodium gallinaceum* in chicks. A series of amides related to phenylpantothenone were reported by Lutz et al. (1947). In this study, (—)-phenylpantothenone was found inactive against avian malaria. This compound corresponds to the unnatural form of panto-

thenic acid. The caproic acid derivatives of β -aminopropiophenone were inactive as antimalarials. Against *Lactobacillus casei*, however, these caproic acid compounds showed some activity. This is particularly interesting because in these compounds both the pantoic acid and the β -alanine moiety of the pantothenic acid molecule are changed. dl-N-Pantoyl- β -phenylethylamide was inactive as an antimalarial but weakly active against *casei*. p-Chlorophenylpantothenone was found to be more active than phenylpantothenone against malaria. Other members of the series synthesized by this group are presented in the general chart of pantothenic acid displacers.

Cantrell (1949) recently published details of work with pantoyltauramide in which he used dosages of 400 mg. per kilogram given to chickens on the sixth day of the infection. The antimalarial action of the compound was completely nullified by 25 mg. per kilogram of pantothenic acid given with the displacers.

Trichomoniasis

Demonstration of pantothenic acid as an essential metabolite for *Trichomonas vaginalis* (Kupferberg et al., 1948) led to the investigation of pantothenic acid displacers in the chemotherapy of this parasite. (+)- α - β -Dihydroxy- β , β -dimethyl-N-(2-(phenylsulfinyl) ethyl) butyramide; d-(+)-(pantoyltauryl)-p-anisidide; N-(2-benzylethyl)- α , γ -dihydroxy- β , β -dimethylbutyramide; (+)- α , γ -dihydroxy- β , β -dimethyl-N-(2-(phenylmercapto)-ethyl)-butyramide were effective in vitro against *Trichomonas vaginalis*. Studied in the same way, eight other pantothenic acid analogues were found inactive. *Trichomonas foetus* and *Trichomonas gallinae* were equally sensitive in vitro to the displacers listed as positive for *Trichomonas vaginalis*. Calcium pantothenate completely nullified the action of the displacers found active. In vivo in monkeys and in a series of six patients the compounds were found ineffective, but inasmuch as administration of the drug was by mouth and vaginally in monkeys and vaginally only in the patients (1 mg. of the displacer twice daily over a period of only two weeks) it does not seem that an adequate test of the possible clinical efficacy of these drugs has been made. The pantothenic acid level of vaginal secretions in patients with trichomoniasis would have to be ascertained with subsequent adjustment of dosage schedule. The safety of such trials was demonstrated by Singher et al. (1948) who gave 1 mg. doses of the same drug vaginally twice daily for ten days to rats and noted no irritation of the vaginal mucosa.

Pantothenic acid displacers should be tried in every clinical state in which the invading parasite or the aberrant cell is known to require this vitamin. It is relatively easy to ascertain the differential requirements of normal cells by contrast. The potentiality of synthesis of pantothenic acid by the prime

parasite or by a secondary parasite must be considered, and it is therefore logical to choose those metabolite analogues which both displace formed pantothenic acid and prevent its formation. These agents should offer the greatest potentialities for clinical application.

Recapitulation

Metabolite analogues of pantothenic acid have been synthesized in great numbers. They can be roughly placed in three categories: those with β -alanine moiety modification, those with pantoic acid moiety modification, and those modified in both pantoic acid and β -alanine units.

Of the first type, pantoyltaurine is a typical example. The mode of action of pantoyltaurine and probably of all analogues of this series has its origin in the inhibition of the metabolism of pantothenic acid which prevents the formation of chemicals normally required for growth, specifically and possibly solely, coenzyme A. Pantoyltaurine is effective as a growth inhibitor only against those organisms requiring preformed pantothenic acid; its action is completely reversed by the vitamin. Resistance to the action of pantoyltaurine develops in various bacterial strains; the probable explanation of this lies in qualitative variation in receptor position on enzymes. Both primary inhibitors (pantothenic acid and its metabolic products) and secondary inhibitors (amino acids) are known for the activity of these analogues.

Metabolite analogues of pantothenic acid with β -alanine moiety modification are active in preventing the growth of those microorganisms which require preformed pantothenic acid and which metabolize this factor. This strongly suggests that the incorporation of pantothenic acid into the coenzyme A molecule is in some manner associated primarily with the β -alanine portion of the vitamin.

Many compounds of the second category (pantoic acid moiety modification) have been synthesized, for example ω -methylpantothenic acid. It is active only against microorganisms requiring preformed pantothenic acid and its activity is completely nullified by the nutritive. Some members of this series such as γ -hydroxy-n-butyryl- β -alanide may act by preventing the synthesis of pantothenic acid, others such as ω -methylpantothenic acid by a mechanism essentially similar to that of pantoyltaurine, except that the reaction sites differ.

Metabolite analogues of pantothenic acid with pantoic acid moiety modification in general seem to be effective in preventing the synthesis of the vitamin. The implication contained in this finding is that the prime point of reaction in this mechanism of synthesis is the pantoic acid moiety which must fit into some receptor site. Pantoic acid analogues may in themselves prevent the coupling of pantoic acid with β -alanine (Cheldelin and Schink, 1947).

Hybrid molecules such as *m*-methylpantoyltaurine have been produced and on study found to act as typical displacers, being completely reversed by pantothenic acid. Certain molecules structurally dissimilar to pantothenic acid, salicylic and mandelic acids, have been reported as interfering with the normal metabolism of the nutritive and exerting an inhibitory effect on bacterial growth by virtue of this.

Until recently all metabolite analogues for pantothenic acid tested failed to interfere with the action of preformed coenzyme A (Novelli and Lipmann, 1948; Martin et al., 1950) in the specific sense, although salicyloyl- β -alanide inhibited the action of choline acetylase. Then, Cohen et al. (1949) demonstrated the inhibition of the formation of coenzyme A by pantoyltaurine and the inhibition of the action of preformed coenzyme A by d-(2-(pantoylamino)ethylsulfono)-4-nitroanilide.

Thus, at least three phases of pantothenic acid metabolism can be inhibited: first, the formation of pantothenic acid by molecules related to pantoic acid; second, the formation of coenzyme A by molecules such as pantoyltaurine; and third, the action of coenzyme A by molecules such as the sulfono-4-nitroanilide. This latter action of pantothenic acid analogues opens new vistas for further work as it represents the logical approach to the inhibition of acetylcholine formation in vivo with important potential clinical applications.

Several different metabolite analogues of pantothenic acid have been established as being effective chemotherapeutic agents against bacterial infections in vivo. While it is controversial, the present author believes that adequate demonstration of the production of a pantothenic acid deficiency state by metabolite analogues has been recorded. Pharmacological investigations have been few and the potentialities in this sphere are great particularly in view of the possible synthesis of many agents which will inhibit the action of coenzyme A functioning as choline acetylase.

The initial phases of the possible application of this class of analogues as antimalarials have been recorded. Pantoyltauramide-4-chlorobenzene is four times as potent as quinine in a standard four day trophozoite-induced infection and 16 times as active in blood-induced infections of seven day duration in chicks. This may be due to inhibition by the compound of the activity of coenzyme A.

While approximately 100 different metabolite analogues of pantothenic acid have been synthesized, few have been given more than a superficial examination in biological systems. Pantoyltaurine has been studied more intensively than any of the others and yet we know nothing of significance about the pharmacology of this agent. It is even controversial whether or not it is capable of inducing pantothenic acid deficiency in the mouse. It may well be true that the most important members of this metabolite analogue group are the coenzyme A inhibitors and we have to date only one

representative molecule. The use of three different types of displacers together will be interesting; it should result in prevention not only of pantothenic acid synthesis, but of incorporation of the vitamin into coenzyme A, and of the action of coenzyme A. The goal is to stop pantothenic acid metabolism and it seems probable that this can be done only by blocking all channels.

Another interesting point for speculation comes in consideration of the speed of turnover or utilization of coenzyme A and in quantitative requirements of cells for this material. Knowledge of these factors will greatly facilitate the rational approach to the application of metabolite analogues of pantothenic acid. The ability of the animal to utilize coenzyme A as a growth factor and the failure of microorganisms in many instances to do so may open a field of chemotherapy with analogues designed to inhibit the action of the coenzyme. A possible complication lies in the potentiality that bacteria are unable to utilize coenzyme A for growth purposes due to a failure of diffusion through the cell. If this is true, analogues designed as inhibitors of coenzyme A might be of such molecular proportions as to be nondiffusible.

It can be stated unequivocally that the application of pantothenic acid displacers in the world of biochemistry and medicine has not yet been given proper study. It is to be hoped that the future will see this situation corrected.

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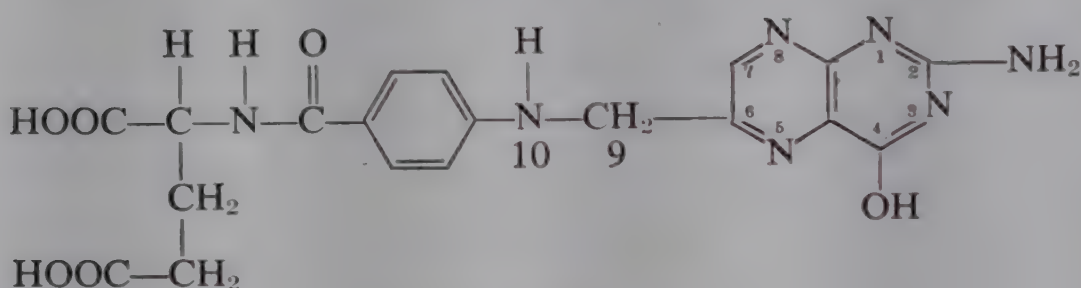
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Chapter 12

METABOLITE ANALOGUES OF PTEROYLGLUTAMIC ACID



Pteroylglutamic acid
N-(4-(((2-amino-4-hydroxy-6-pteridyl)methyl)amino)benzoyl)
glutamic acid

1. General Studies of Analogues in Bacterial Systems.
 - a. Metabolite Analogues with Pteridyl Ring Modification or Replacement.
 - b. Metabolite Analogues with Modification in the Glutamic Acid Moiety.
 - c. Metabolite Analogues with Modification in the p-Aminobenzoic Acid Moiety.
2. Enzymes and Folic Acid Displacers.
3. Pharmacology of Folic Acid Analogues.
4. Pteridines as Folic Acid Antagonists.
5. Purine, Pyrimidine, and Folic Acid Antagonists.
6. Animal Studies with Folic Acid Analogues.
7. Animal Studies Involving Tumor Chemotherapy with Folic Acid Analogues.
8. Clinical Studies Involving Metabolite Analogues of Folic Acid.
9. Recapitulation.

Folic acid has had a relatively brief career, but an exciting one. It plays a vital role in microbiology, where it has been established that many, if not all, bacteria require the vitamin for growth and reproduction. *Lactobacillus casei* and *Streptococcus lactis* R are particularly sensitive to deficiencies of this factor. Some bacteria synthesize folic acid. In the plant world, the nutrilitic is almost universally present. Folic acid deficiency has been observed under experimental conditions in insects, parasites, chickens, turkeys, rats, guinea pigs, dogs, and monkeys. It is probably essential to most animals for normal hemopoiesis, growth, and health. In man, the part played by folic acid is directed primarily toward the blood forming structures. The vitamin is effective in the macrocytic anemias of nutritional

origin, in the megaloblastic anemias of sprue, of pregnancy, and in that of infancy and childhood. With vitamin B₁₂, it is active in pernicious anemia. Pteroylglutamic acid is linked to the production of nucleic acids through thymine and thymidine. It bears an obvious structural and biological relationship with naturally occurring pterines, such as xanthopterin. The enormous literature on the subject recently reviewed by Jukes and Stokstad (1948) formed the background for the synthesis and subsequent study of molecules designed as metabolite analogues of pteroylglutamic acid.

As a structure for the design of metabolite analogues, it is only necessary to view the parent molecule as consisting of a pteridine grouping linked through p-aminobenzoic acid to a glutamic acid residue. Each of the structural units could be modified or entirely replaced. As will be seen from the following, virtually every potentiality for modification of the basic structure has been utilized, generally successfully, for the production of metabolite analogues. The designs for effective analogues are reviewed at the end of the first section. The type of metabolite analogue discussed initially is that in which structural modifications are made in the pteridyl portion of the molecule or in which the entire pteridyl radical is replaced by some structurally similar double ring unit.

General Studies of Analogues in Bacterial Systems

Metabolite Analogues with Pteridyl Ring Modification or Replacement

The first compound to be described was methylfolic acid, designated as N-(4(((2-amino-4-hydroxy-7-methyl-6-pteridyl) methyl) amino) benzoyl)-d-(—)glutamic acid. Martin et al. (1947) demonstrated that the molecule was an effective displacer of folic acid when *Streptococcus faecalis* (lactis) R was the test organism. The ratio of inhibitor to metabolite was approximately 150. This compound in the l-(+) form has been variously designated as x-methylfolic acid and crude folic acid antagonist, based upon divergence of opinion as to its structure and purity. Franklin et al. (1947) tested the microbiological effect of l-(+)methylfolic acid and found that it, too, antagonized the growth effect of folic acid on *Streptococcus faecalis* R as well as on *Lactobacillus casei*. As with the isomer, the action was completely reversible. The inhibition ratio for *Streptococcus faecalis* R was 20:1 and for *Lactobacillus casei* 1000:1.

An attempt to delineate the prime point of action of methylfolic acid led Martin et al. (1947a) to study the reversal of its action on *Staphylococcus aureus*. Methylfolic acid was counteracted by pteroylglutamic acid, pterioic acid, and p-aminobenzoic acid but not by p-aminobenzoyl-l-(+) glutamic acid, indicating that methylfolic acid interferes with the synthesis of pteroylglutamic acid at the stage of pterioic acid formation and at the stage of union

with glutamic acid. It had the further capacity to displace formed pteroylglutamic acid; thus, it represents another displacement compound incorporating into one molecule the capacity to inhibit the formation of and displace a given metabolite. As with a sulfonamide, methylfolic acid interferes with the incorporation of p-aminobenzoic acid into pteroylglutamic acid. The counteraction of methylfolic acid by sulfathiazole was demonstrated and was interpreted to indicate similarity of structure causing a mutual interference which superseded that of interference with the metabolism of pteroylglutamic acid. These findings and the interpretations placed on them are applicable only to *Staphylococcus aureus*.

By the application of inhibition analysis and the use of methylfolic acid, Bond et al. (1949) have obtained evidence for their folinic acid. This acid was 10 to 100 times as active as folic acid in preventing the toxicity of methylfolic acid for *Streptococcus faecalis* R. These observations grew out of the work done by this same group (Rogers and Shive, 1948) when they used methylfolic acid in a medium toxic to *Lactobacillus casei* and observed that liver extracts prevented the toxicity in a competitive manner and that the activity of the liver extract was 15 times as great as could be accounted for on the basis of the folic acid content.

Aminopterin (4-aminopteroylglutamic acid) (N-(4-(((2,4-diamino-6-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid) (Fig. 1) was the second metabolite analogue of folic acid to be synthesized (Seeger et al., 1947). The inhibition ratio for half maximum inhibition of the growth of *Strep-*

Table I

INHIBITION OF *Streptococcus faecalis* R WITH 4-AMINOPTEROYLGLUTAMIC ACID

Metabolite	Concentration of Metabolite γ per ml.	Inhibition Index*
Pterioic acid	0.004	0.99
" "	0.04	0.32
" "	0.4	0.09
Pteroylglutamic acid	0.003	2.66
" "	0.03	0.19
" "	0.3	0.07
Pteroyl-γ-glutamylglutamic acid	0.005	4.30
" "	0.05	0.33
" "	0.5	0.10
Pteroyl-γ-glutamyl-γ-glutamylglutamic acid	0.07	0.49
" "	0.7	0.08
" "	7.0	0.007

* Ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition. (From Oleson et al., *J. Biol. Chem.*, **175**, 359, 1948.)

Staphylococcus faecalis R is 1.9, 0.7, and 0.4 at concentrations of pteroylglutamic acid of 0.003, 0.005, and 0.01 microgram per 10 ml. respectively. Subsequent studies of aminopterin indicated that its action was not of a strictly competitive nature (Oleson, et al. 1948). The compound was most effective as an inhibitor of the growth promoting action of pteroyltriglutamic acid and pterioic acid. Table I indicates this fact together with the variability of inhibition index with concentration.

The reversal by thymidine of some phases of the toxicity of aminopterin not reversed by folic acid has been reported (Franklin et al., 1949; Saubers, 1949) and interpreted as indicating the involvement of folic acid in the formation of thymidine. 4-Amino-5-carboxyamidoimidazole, metabolic precursor of hypoxanthine, accumulates during the growth of *Escherichia coli* in the presence of 4-aminopteroylglutamic acid (Woolley and Pringle, 1950). This finding reflects the production of a folic acid deficiency with consequent failure of purine formation.

Several modifications of the 4-amino structure have been prepared, including 4-aminopteroylaspartic acid (Hutchings et al., 1949); 4-amino-10-methylpteroylglutamic acid and the corresponding pterioic acid (Franklin et al., 1949a; Seeger et al., 1949). These agents are effective metabolite displacers in bacterial systems, differing quantitatively but not qualitatively from the action of the parent 4-aminopteroylglutamic acid.

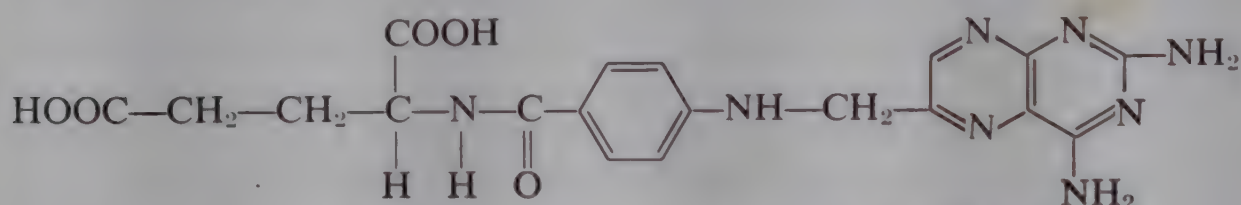
The pteridyl portion of the pteroylglutamic acid molecule is modified in the 9-methylpteroylglutamic acid derivatives which are once again effective antagonists (Hultquist et al., 1949). This molecule has a 9-methyl radical which is the equivalent of a modification of the 6-methyl substituent to a 6-ethyl on the pteridyl ring. Still another type is oxyfolic acid (Martin et al., 1948) in which the 2-amino group is replaced by a hydroxyl. This molecule was inactive in bacterial test systems but active as an inhibitor of dopa decarboxylase and in the production of a pharmacological activity associated with metabolite displacers of the vitamin (Martin et al., 1948a). An interesting pteridyl moiety modification which resulted in an effective metabolite analogue was that prepared by Woolley and Pringle (1948), and designated 2-amino-4,7-dihydroxypteridine-6-carboxylyl-p-aminobenzoylglutamic acid. Finally, a 7-pteridyl compound has been reported (Avakian et al., 1950).

Another major category of displacing agents has been produced by replacement of the entire pteridyl nucleus. One of the first attempts to do this led to the synthesis of N-(4-((4-quinazoline)amino)benzoyl)glutamic acid (Martin et al., 1947b) which molecule possessed stimulatory rather than inhibitory action on *Streptococcus faecalis lactis* R. A similar molecule, quinoxaline-2-carboxylyl-p-aminobenzoylglutamic acid, was an effective metabolite antagonist (Woolley and Pringle, 1948). Replacement of the pteridyl moiety by benzimidazole resulted in a molecule with stimulatory

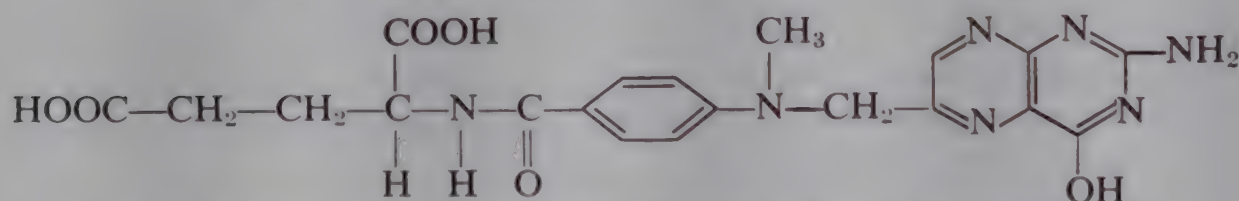
rather than inhibitory activity (Edwards et al., 1948) until the p-amino-benzoic acid moiety was modified by replacing the carboxyl with a sulfonic acid unit. King et al., (1948; 1949) report the carboxyl compound as having a low order of inhibitory activity. The 5-chloro analogue was more powerful using *Lactobacillus casei* as testing system.

*Metabolite Analogues with Modification
in the Glutamic Acid Moiety*

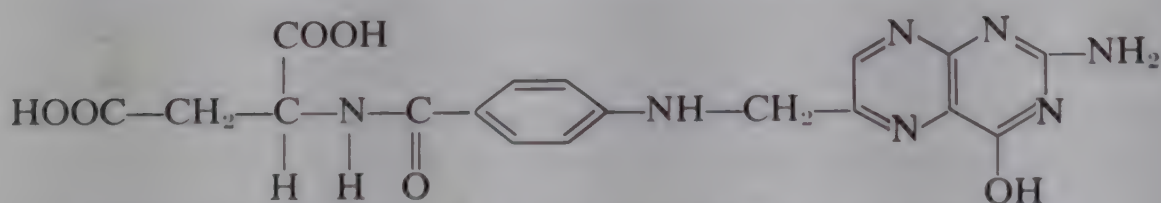
Pteroylaspartic acid (Fig. 1) was the first compound of the series to be described in which the glutamic acid moiety has been modified (Hutchings et al., 1947). The molecule proved to be a typical inhibitor of the competitive type. One point of distinction from 4-aminopteroylglutamic acid was the ineffectiveness of pteroylaspartic acid in interfering with the metabolism involving pteroylglutamic acid or derivatives in *Escherichia coli*.



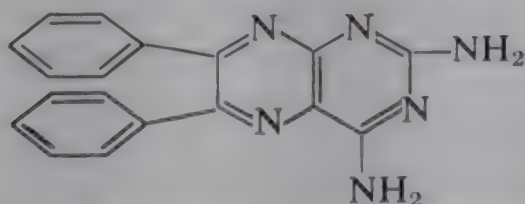
4-Aminopteroylglutamic acid; aminopterin; N-(4-(((2,4-diamino-6-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid



N10-Methylpteroylglutamic acid; N-(4-(N-((2-amino-4-hydroxy-6-pteridyl)methyl)-N-methylamino)-benzoyl)-glutamic acid



Pteroylaspartic acid; N-(4-(((2-amino-4-hydroxy-6-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid



2,4-Diamino-6,7-diphenylpteridine

FIG. 1. Metabolite analogues of pteroylglutamic acid.

Pteroyl derivatives of alanine, ϵ -aminocaproic acid, aminomalonic acid, isoleucine, phenylalanine, sarcosine, serine, threonine, tryptophan, valine, β -alanine, cystine, and methionine have been prepared in the crude form (Wright et al., 1949). Of these, pteroylalanine, pteroyl- β -alanine, pteramidomalonic acid, and 4-aminopteroylserine were purified. Smith (1950) reports that this entire group of analogues is either inactive or only weakly effective. Two additional members of this series, namely the glycine and the methionine sulfoxide analogues, have been found effective metabolite displacers (Martin et al., 1948a; Woolley and Pringle, 1948).

*Metabolite Analogues with Modification
in the p-Aminobenzoic Acid Moiety*

Considering the p-aminobenzoic acid moiety of the folic acid structure, it is apparent that it might be modified by substitution on the amino group, by substitution of a sulfonic unit for the carboxyl or by replacement or modification of the benzene ring. All three types of displacer have been produced.

The first is typified by N10-methylpteroylglutamic acid (Fig. 1) which carries a methyl substituent on the amino group of the p-aminobenzoic acid moiety (Cosulich and Smith, 1948); the second by pteroylsulfoglutamic acid (Viscontini and Meirer, 1949; Gavard and Viscontini, 1949) in which the carboxylic group of the p-aminobenzoic acid is changed to a sulfonic unit; and the third by 2-chloro-4-((N-(2-amino-4-hydroxy-6-pteridyl)methyl)amino)benzoic acid (Martin et al., 1948a) in which the benzene nucleus of the p-aminobenzoic acid moiety bears a chlorine substituent. All three types were typical metabolite analogues.

In summary, each moiety of the pteroylglutamic acid molecule has been modified in many ways with consequent production of effective metabolite antagonists. There is little of a fundamental nature distinguishing the action of one type from that of another, differences being quantitative rather than qualitative. It seems probable, however, that future work will reveal basic mechanisms of action. The pteridyl ring can be modified by changing 4-hydroxy to 4-amino; 6-methyl to 6-ethyl; 2-amino to 2-hydroxy; 7-hydrogen to 7-methyl and by shifting the point of attachment of the p-aminobenzoic acid bridge thus creating a 7-pteridyl replacing a 6-pteridyl. It is even possible to replace the entire pteridyl nucleus by benzimidazole or quinoxaline and gain displacer molecules. The p-aminobenzoic acid bridge can be modified by replacing the carboxyl by sulfonic; by modifying the amino grouping through a methyl substituent; and by modifying the benzene nucleus by substitution of chlorine for hydrogen. Glutamic acid may be replaced by aspartic acid or glycine with the desired result. It is clear that additional potentialities exist for design of metabolite displacers.

Bacteria have been classified on the basis of their folic acid nutrition into

three categories (Jukes and Stokstad, 1948); those which require preformed folic acid such as *Streptococcus faecalis* R and *Lactobacillus casei*, those which can both synthesize folic acid if needed or use preformed vitamin such as *Streptococcus faecalis* R and finally those which do not require preformed pteroylglutamic acid and which probably cannot utilize the preformed vitamin. One would imagine that as with pantothenic acid so will the situation reveal itself in connection with the pteroylglutamic acid displacers. Three categories of analogues will probably be found; those which prevent the synthesis of pteroylglutamic acid, those which prevent the synthesis of the pteroylglutamic acid enzymes, and those which prevent the action of these enzymes.

There are many examples of folic acid displacers, e.g., pteroylaspartic acid, which act against those bacteria requiring preformed folic acid by virtue of a capacity to prevent the formation of folic acid enzymes. There are recorded examples of displacers which act against bacteria which do not require preformed pteroylglutamic acid and can synthesize this factor. Thus, 4-aminopteroylglutamic acid is effective against *Escherichia coli* and methylfolic acid is active against *Staphylococcus aureus*.

The role of folic acid in enzyme systems is not clear. It seems probable that in the choline oxidase system folic acid is incorporated into the enzyme as a prosthetic grouping and that this enzyme formation is inhibited by 4-aminopteroylglutamic acid. This may be the case with dopa decarboxylase but it has not been established. Sulfonamides and possibly certain pteridines can be regarded as metabolite antagonists preventing the synthesis of folic acid. The one proposed category of analogues yet to be demonstrated is that series interfering with the action of a preformed pteroylglutamic acid enzyme.

Enzymes and Folic Acid Displacers

A study of the inhibitory action of metabolite antagonists of folic acid on enzyme systems in vitro would be fundamental to an understanding of the true mechanism of their action in vivo. The first report on this phase (Martin and Beiler, 1947c) dealt with the action of dopa decarboxylase. Both methylfolic acid and pteroylaspartic acid were effective inhibitors of the enzyme. The action was reversible. At a concentration of 30 micrograms per ml., the methylfolic acid compound caused an inhibition of 25 per cent. Folic acid added at 10–100 times the concentration of the methylfolic completely prevented the inhibitory action. Methylfolic acid did not inhibit the action of tyrosine decarboxylase except at such high concentrations as to indicate the lack of specificity. This inhibitory action by metabolite antagonists of folic acid has led to the use of these chemicals in vivo for the reduction of blood pressure (Martin et al., 1947d, 1948a).

Extending the series of antagonists used to determine some phase of cor-

relation between structure and capacity to inhibit dopa decarboxylase, Martin and Beiler (1948b) observed four general classes of displacers: (1) compounds which differed by the presence of an additional or substituted group on the pteridyl part of the molecule; (2) compounds in which the glutamic acid moiety is modified or omitted; (3) a combination of (1) and (2); and (4), a compound in which the pteridyl grouping is replaced. The results showed that the most effective displacers of the series tested had a substituent on the pteridyl part of the molecule in combination with an amino acid. Substitution of aspartic acid for glutamic did not change significantly the inhibitory effect. Substitution of aspartic acid for glutamic without the presence of the substituent on the pteridyl ring, or complete removal of the amino acid, resulted in the formation of less effective metabolite displacers. Replacement of the pteridyl ring with a quinazoline ring did not produce an effective displacing agent. A series of approximately 30 compounds designed to be displacers of folic acid were tested as inhibitors of dopa decarboxylase and an attempt was made to correlate this activity with blood pressure changes (Martin et al., 1948a).

Methylfolic acid proved to possess no activity against histidine decarboxylase (Beiler et al., 1949) at levels as high as 1 mg. per ml. In fact, the molecule seemed to stimulate the action of the enzyme. This observation was theoretically explained on the basis of the probability that folic acid is present in some other enzyme which attacks histidine, and that this is inhibited by the vitamin antagonist. Such action would, in effect, raise the concentration of histidine available to the histidine decarboxylase, and thus increase the activity of the enzyme.

Potentialities for enzymatic activities of metabolite analogues of folic acid arise in connection with each of the enzyme systems for which folic acid is a substrate or the action of which is modified by folic acid. Kalckar and Klenow, (1948) noted the powerful inhibition of milk xanthopterin oxidase by synthetic pteroylglutamic acid. Liver xanthopterin oxidase was also inhibited, as was xanthine oxidase. Uricase was unaffected. It is probable that the enzymes inhibited by folic acid would be stimulated by metabolite antagonists just as histidine decarboxylase is.

An enzymatic transformation of folic acid itself was recorded (Kalckar and Klenow, 1948a) and one may anticipate an inhibition of this metabolic conversion by the displacers. In December 1949 a report appeared (Dinning, Keith, and Day, 1949) which demonstrated that pteroylglutamic acid functions in the choline oxidase enzyme system as a constituent of the prosthetic group. Aminopterin was found to be a specific inhibitor of choline oxidase *in vivo*.

The role of pteroylglutamic acid as the prosthetic group of an enzyme or enzymes or as a coenzyme concerned with the synthesis of thymine, purines, and derivatives was originally proposed by Stokes (1944). Further

evidence supporting this contention was presented by Lampen and Jones (1947), Petering and Delor (1949), Prusoff et al. (1948) and others. It remained for Rogers and Shive (1948) to establish the capacity of folic acid analogues in the inhibition of the enzyme or coenzyme involved when they reported that the antibacterial index of methylfolic acid for *Lactobacillus casei* was 30 in the absence of purines and pyrimidines and 1000 in the presence of these metabolites. The culmination of efforts along these lines came with announcements that the inhibitory effect of methylfolic acid and to a certain extent of aminopterin could be reversed by thymidine (Franklin et al., 1949; Sauberlich, 1949; Shive et al., 1948).

Thus, metabolite antagonists of folic acid are inhibitors of dopa decarboxylase, choline oxidase, and enzymes involved in purine and pyrimidine metabolism. They are not active against histidine decarboxylase. The potentiality is that the inhibitors will block enzymatic mechanisms in which folic acid plays a role, whether as substrate, prosthetic group, or cofactor.

Pharmacology of Folic Acid Analogues

Very little study has been given to the pharmacological behavior of metabolite antagonists of folic acid, unless one considers the term "Pharmacology" in its broadest aspects.

In 1947, Martin et al. (1947d) reported the hypotensive action of 7-methylfolic acid in dogs. A dosage level of 5 mg. per kilogram dropped blood pressure by 20–40 mm. Tachyphylactic phenomena developed. 100 mg. of the methylfolic acid per kg. caused death by respiratory arrest within four minutes. Subsequently, details of this work were reported (Martin, et al., 1948a) involving a series of displacing agents produced by modification of the pteridyl ring, the p-aminobenzoic acid moiety, or the glutamic acid moiety. Many displacers were included which were changed at several points. In general, there was a rough correlation between the capacity of a given molecule to inhibit dopa decarboxylase and its ability to lower blood pressure in the dog, but such correlation was not absolute. 4-(((2-Amino-4-hydroxy-6-pteridyl)methyl)amino)phenol was a powerful blood pressure depressant but was inactive in the in vitro enzyme system against dopa decarboxylase.

For hypotensive action, the pterioic acid analogues were superior to the pteroylglutamic acid analogues. Metabolite inhibitor ratios for the three systems studied showed that for the *Streptococcus faecalis* system, the ratio was 1 to 10; in the dopa decarboxylase system, the ratio was 0.1 to 0.01; in the dog, the ratio was approximately 0.1 to 0.01. In other words, a concentration of folic acid from 10 to 100 times that of the displacer nullifies the displacer action. Two hundred and fifty milligrams of folic acid per kilogram nullified the hypotensive action of 10 mg. of displacer. Tachyphylactic phenomena developed with marked speed as shown in Table II.

Table II*

"TACHYPHYLACTIC" PHENOMENA ASSOCIATED WITH THE PHARMACOLOGICAL ACTION OF PTEROYLGLUTAMIC ACID DISPLACING AGENTS

Compound	Blood Pressure Effect of Five Doses (5 mg./kg. each) Given in Sequence to a Single Animal				
	1	2	3	4	5
Oxypteroic acid	— 70 mm. 10 min.	— 60 mm. 10 min.	— 50 mm. 8 min.		
	— 62 mm. 15 min.	— 50 mm. 10 min.	— 42 mm. 10 min.	— 28 mm. 10 min.	— 24 mm. 10 min.
	— 110 mm. 10 min.	— 86 mm. 10 min.	— 82 mm. 10 min.	— 44 mm. 10 min.	
Methylpteroic acid	— 60 mm. 5 min.	— 20 mm. 3 min.	No effect	No effect	No effect
	— 74 mm. 30 min.	— 28 mm. 20 min.	No effect	No effect	
	— 60 mm. 10 min.	— 16 mm. 3 min.	No effect	No effect	No effect

* From Martin et al., *Am. J. Pharmacy*, **120**, 189, 1948.

The oral administration of methylfolic acid produced no effect on blood pressure in doses as high as 500 mg. per kilo.

The hypotensive action of folic acid displacers has not been reported clinically. The dosages used in the treatment of the leukemias are too low to produce blood pressure effects if one transfers the required levels for the dog to consideration of dosage needed in man. It is highly probable that certain metabolite antagonists, for example 4-aminopteroylglutamic acid, will be much more specifically active against the bone marrow, while one such as oxyfolic acid, the 2,4-dihydroxy compound, may be effective as a blood pressure depressant. It has already been demonstrated that there is no 100 per cent correlation in specificity of molecules tested in different biological systems. It is unfortunate that to date so few of the different antagonists for folic acid have been screened; but this will come.

Pteridines as Folic Acid Antagonists

The demonstration that synthetic pteridines are specific metabolite antagonists for folic acid (Daniel et al., 1947; Mallette et al., 1947; Weygand et al., 1948) makes a brief review of these compounds important to an understanding of this displacer group. Daniel et al., (1947) tested four pteridines, namely, 2-amino-4-hydroxypteridine; 2,4-dihydroxypteridine; 2-mercapto-4-hydroxypteridine; and 2,4-diamino-6,7-diphenylpteridine. Of these only the last was active. The antibacterial action of this compound was reversed by folic acid. The molecule possessed unique capacity for the inhibition of growth in bacteria which synthesize folic acid as well as those requiring the vitamin preformed. Actually, in any search for an antibacterial

this combined action is desirable as it should prevent the development of resistance in the bacteria. The phenomenon of resistance is generally due to synthesis of the metabolite essential to growth.

Extending their work, Daniel and Norris (1947) reported the effectiveness of pteridines against *Escherichia coli* and *Staphylococcus aureus*. The pteridines were 2,4-diamino types and acted synergistically with sulfonamides in inhibiting the growth of microorganisms that synthesize folic acid.

Table III
METABOLITE ANALOGUES OF PTEROYLGLUTAMIC ACID

Compound	Common Name	Activity in at least One Test System	Reference
<i>Pteridyl Moiety Modification</i>			
N-(4-(((2-Amino-4-hydroxy-7-methyl-6-pteridyl)-methyl)-amino)-benzoyl)-d(—)-glutamic acid	d(—)Methyl-folic	+	Martin, et al., 1947
N-(4-(((2,4-Diamino-6-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid	Aminopterin(4-aminopteroyl-glutamic acid)	+	Seeger, Smith, and Hultquist, 1947
N-(4-(((2,4-Diamino-6-pteridyl)-methyl)-amino)-benzoyl)-aspartic acid	4-Amino-pteroyl-aspartic acid	+	Hutchings et al., 1949
N-(4-(((2,4-Diamino-6-pteridyl)-methyl)-N-methylamino)-benzoyl)-glutamic acid	4-Amino-10-methyl-pteroyl-glutamic acid; A-meth-opterin	+	Franklin et al., 1949a; Seeger et al., 1949
N-(4-(((1-(2-Amino-4-hydroxy-6-pteridyl)-ethyl)-amino)-benzoyl)-glutamic acid	9-Methyl-pteroyl-glutamic acid	+	Hultquist et al., 1949
N-(4-(((2,4-Dihydroxy-6-pteridyl)-methyl)-amino)-benzoyl) 1(+)-glutamic acid	Oxyfolic acid	+	Martin et al., 1948
2-Amino-4,7-dihydroxy pteridine-6-carboxyl-p-aminobenzoylglutamic acid		+	Woolley and Pringle, 1948
N-(4-(((2-Amino-6-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid	4-Desoxy-pteroyl-glutamic acid	+	Swendseid et al., 1948
N-(4-(((2-Amino-4-hydroxy-7-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid	7-Pteroyl-glutamic acid	+	Avakian et al., 1950

Table III—(Continued)

<i>Compound</i>	<i>Common Name</i>	<i>Activity in at least One Test System</i>	<i>Reference</i>
<i>Pteridyl Moiety Replaced</i>			
N-4-((4-Quinazoline)-amino)-benzoyl]-glutamic acid		Stimulatory —	Martin et al., 1947b
Quinoxaline-2-carboxyl-p-aminobenzoylglutamic acid		+	Woolley and Pringle, 1948
N-[4-((2-Benzimidazolyl)-methyl)-amino)-benzoyl]-glutamic acid		Stimulatory —	Edwards, et al., 1948; King et al., 1948, 1949
N-[4-((2-Benzimidazolyl)-methyl)-amino)-benzene-sulfonyl]-glutamic acid		+	Edwards, et al., 1948
N-[4-((2-(5-Chlorobenzimidazolyl)-methyl)-amino)-benzoyl]-glutamic acid		+	King et al., 1948, 1949
<i>p-Aminobenzoic Acid Moiety Modifications</i>			
N-(4-(N-(2-Amino-4-hydroxy-6-pteridyl)-methyl)-N-methylamino)-benzoyl)-glutamic acid	N ¹⁰ -Methyl-pteroylglutamic acid	+	Cosulich and Smith, 1948
Also N ¹⁰ -ethyl-and butyl-and crude N ¹⁰ -carboxymethyl-, benzyl-, phenacylpteronic acid and N ¹⁰ -phenacyl-pteroylglutamic acid		Weakly +	" "
2-Chloro-4-((N-(2-amino-4-hydroxy-6-pteridyl)-methyl)-amino)-benzoic acid	Chloropteroic acid	+	Martin et al., 1948a
Pteroylsulfo-L(+)-glutamic acid		+	Viscontini and Meirer, 1949
<i>Glutamic Acid Moiety Modifications</i>			
N-(4-((2-Amino-4-hydroxy-6-pteridyl)-methyl)-amino)-benzoyl)-aspartic acid	Pteroylaspartic acid	+	Hutchings et al., 1947
4-Aminopteroyltri-glutamic acid and similar peptides		+	Oleson, 1950
4-Amino-9-methylpteroyl-glutamic acid		+	Oleson, 1950
4-Amino-9,10-dimethyl-pteroylglutamic acid		+	Oleson, 1950
4-Aminopteroyl- α -alanine		+	Oleson, 1950
4-Aminopteroylthreonine		+	Oleson, 1950

Table III—(Continued)

Compound	Common Name	Activity in at least One Test System	Reference
<i>Glutamic Acid Moiety Modifications</i>			
9,10-Dimethylpteroylglutamic acid		+	Oleson, 1950
6-Methyl-7-pteroylglutamic acid		+	Oleson, 1950
4-Aminopteroylserylglutamic acid		+	Oleson, 1950
4-Dimethylaminopteroylglutamic acid		—	" "
N-10-Nitropteroylglutamic acid		—	" "
Dichloropteroylglutamic acid		—	" "
4-Aminopteroylsulfoglutamic acid	S-Aminopterin	+	Southern Research Institute, 1949
N (3-(((2,4-Diamino-6-pteridyl)-methyl)-amino)-benzoyl)-glutamic acid	M-Aminopterin	+	Southern Research Institute, 1949
Corresponding amino acid deriv. alanine, α -aminocaproic, aminomalonic, isoleucine, phenylalanine, sarcosine, serine, threonine, tryptophan, valine, β -alanine, cystine, and methionine		All of Wright series reported negative —	Wright et al., 1949; Smith, 1950
Pteroylmethionine sulfoxide		+	Woolley and Pringle, 1948
Pteroyl d(—)-glutamic acid		—	Oleson, 1950
Pteroylserylglutamic acid		—	Oleson, 1950

The authors state that "the synergism shown by sulfonamide and pterine is one in which there are two inhibitors, one of which prevents the functioning of an enzyme active in the synthesis of folic acid, and the other of which appears to prevent the formation of an enzyme in which folic acid probably acts as the prosthetic group." This offers a possible explanation but not necessarily the only one. Both the sulfonamide and the pteridine might function against the enzymatic system producing folic acid.

Some 30 pteridines and pyrimidines were tested in chicks for their capacity to increase growth and hemoglobin formation (Daniel, et al., 1948). 2-Amino-4-hydroxy-6,7-diphenylpteridine and 2-amino-4-hydroxy-6,7-dimethylpteridine inhibited growth and hemoglobin formation. The inhibitory action was reversed by folic acid. The pteridines and pyrimidines showing a positive action were active only in the presence of suboptimal

levels of folic acid. The 2,4-diaminopterine which these investigators had found active in bacterial systems were inactive when tested in vivo on the chick. The selective action should make 2,4-diaminopteridines extremely effective antibacterials. In vivo antibacterial studies with these agents have not yet been reported.

The activity of the 2-amino-4-hydroxy compounds and the inactivity of the 2,4-diamino compounds in vivo demonstrates once again the failure of metabolite antagonists to function equally in various test systems. This very fact magnifies the extent of the problem facing medical science, i.e., that of testing all metabolite antagonists in those systems in which it is desired to bring about a given effect rather than to assume from bacterial studies, let us say, that a given agent would not be effective in the human being. The reasoning which assumes that the production of a vitamin deficiency state is an all or nothing proposition must give way to an understanding of the complexity of the situation. Another group of investigators (Swendseid, et al., 1949) reported work using the same pteridine (2,4-diamino-6,7-diphenylpteridine) in the rat. The action of the 2,4-diamino compound was confined to an effect on the leukocyte system. Thus, once more we see great species variation (chick contrasted to rat) in the activity of metabolite displacers. It is also a point worthy of emphasis to note the broad variations in structure which can produce metabolite antagonists.

There have been several reports of work the goal of which was the application of the pteridines to the chemotherapy of cancer. Gal (1949) noted the toxic effects of 4,6,7-trihydroxy-2-methylpyrimido(4, 5-B) pyrazine and 2,4-diaminopterine. The latter compound produced extensive kidney and liver damage in the rat. A series of pteridines has been reported (Hertz and Tullner, 1949) as affecting a quantitative inhibition of estrogen-induced tissue growth in the female genital tract. Both the stilbestrol treated chick and the estradiol treated ovariectomized rat were used as testing systems. The following pteridines were found active: 2,4-diamino-6,7-dimethylpteridine; 2-amino-4-hydroxy-6,7-diphenylpteridine; 2-amino-4-hydroxy-6,7-di(p-aminophenyl)pteridine; and 2,4-diamino-6,7-diphenylpteridine. Greenberg (1949) tested a series of pteridines for antimalarial activity against *Plasmodium gallinaceum* in the chick. Of the series tested, only 2,4-diamino-6,7-diphenylpteridine was effective. The antimalarial activity of the compound was about the same as that of quinine. The pteridine was markedly potentiated in its antimalarial activity by sulfadiazine. While folic acid inhibited the action, complete reversibility was not demonstrated. The 6,7-dimethyl and the 6,7-dianiline, but not the 6,7-dicarboxylic acid, were effective antimalarials.

Xanthopterin, 2-amino-4,6-dihydroxypteridine, is one of the naturally occurring pteridines. It was not an effective replacer nor a displacer for folic acid as tested by Daniel et al. (1948). It does not seem to have been ex-

tensively investigated as a displacer for folic acid in various testing systems. Xanthopterin was first reported to play a role in hematopoiesis by Tschesche and Wolfe (1937) using rats on a milk anemia diet. Not only xanthopterin but also erythropterin, leucopterin, and guanopterin gave reticulocyte response in these animals. Following the appearance of this work, xanthopterin has been demonstrated to cure fish anemia (Simmons and Norris, 1941), to possess activity in the regeneration of blood cells in dogs (McKibbin, et al., 1942), to counteract anemia induced in rats by diets containing succinylsulfathiazole (Totter and Day, 1943) and to cause reticulocyte responses and increases in red and white cells in monkeys fed on the vitamin-M deficient diet (Totter et al., 1944).

The pharmacology of xanthopterin is of interest, viewed in the light of the possible metabolite displacer action of this pterine. Horlein (1941) reported the lethal dose of xanthopterin in mice as 50 mg./kg. The animals died after some days and the main pathological findings were tubular degeneration in the kidneys. Injection of 20 mg. per kg. intravenous into rabbits caused a decrease in urine output and even anuria. The pterine produced hyperglycemia lasting for several days. This was explained by the degenerative changes in the pancreas. The toxicity of pteridines in rats has been investigated by LaDu et al., (1950). They studied five 4-aminopteridine compounds. 2,4-Diamino-6,7-dihydroxypteridine and 2,4-diaminopteridine interfere with the growth rate of the rat and the latter compound caused crystalline deposits in the renal tubules. None of the compounds studied altered the blood picture. Thus, it is apparent that the pteridines are pharmacologically active toxic chemicals, and the possibility exists that the basis of the activity lies in metabolite displacement.

Evidence to substantiate this concept comes from the work of Hofstee (1949) who investigated the inhibition of the enzymatic oxidation of xanthine and xanthopterin by other pteridines. The conclusion was reached that one enzyme was involved in the oxidation of xanthine and xanthopterin. Xanthopterin inhibited the oxidation of xanthine to 90 per cent when both are present in equimolar concentrations. It was noted that 4-aminopteroylglutamic acid did not inhibit the oxidases. 2,4-Diamino-6,7-dihydroxypteridine is active as an inhibitor of xanthine oxidase, as were some 11 other pteridines. The competitive inhibition of xanthine oxidation by xanthopterin was also studied by Krebs and Norris (1949) who reported that the relative rates of xanthine and xanthopterin oxidation in the presence of milk oxidase was approximately 25:1. Xanthopterin had a lower dissociation constant with respect to the enzyme than did xanthine, and thus acts as a competitive inhibitor of xanthine oxidation. Most recently, Lowry et al. (Lowry, Bessey, and Crawford, 1949) have found that 2-amino-4-hydroxy-6-formylpteridine is a powerful inhibitor of the enzyme which oxidizes xanthine, xanthopterin, and 2-amino-4-hydroxypteridine.

The University of Washington group have made extensive studies of xanthopterin, reporting that it caused an increase in the rate of cell proliferation of both erythrocytes and total nucleated cells in vitro in a bone marrow suspension (Norris and Majnarich, 1948). The action of xanthopterin in rat anemia produced by sulfathiazole was much more rapid than that of pteroylglutamic acid. This is most interesting taken in conjunction with the observation that incubation of a liver suspension with xanthopterin increased the "folic acid" effect of liver (Wright and Welch, 1943). Norris and Majnarich (1948a) studied the action of a series of pteridines on bone marrow cultures. None of the pteridines studied showed as great an acceleration of bone marrow as xanthopterin. Xanthopterin-7-carboxylic acid and 2-amino-4-hydroxy-7-methylpteridine strongly inhibited bone marrow cell proliferation, which action could be reversed by xanthopterin. Tissue culture experiments revealed that xanthopterin increased the rate of cell proliferation in all tissues studied (Norris and Majnarich, 1948b). 2-Amino-4-hydroxy-7-methylpteridine and xanthopterin-7-carboxylic acid inhibited cell proliferation and this action was reversible by xanthopterin. Folic acid had no effect on the tissue cultures studied. In June of 1948, these investigators (Norris and Majnarich, 1948c) reported the observation that xanthopterin strongly inhibited cell proliferation in tissue culture of neoplastic growths. The antixanthopterins stimulated cell proliferation. Xanthopterin and its metabolite antagonists counteracted the effect of each other in equal concentrations. Blood serum from individuals with cancer or leukopenia had the same action in stimulating cell proliferation in tissue culture of human bone marrow.

It is clear that xanthopterin merits a trial in the chemotherapy of cancer despite its toxicity. The lethal dose is around 10 to 50 mg. per kilogram depending on the species under test and 5 micrograms of xanthopterin per ml. inhibited neoplastic tissue cultures. For a 70 kilogram adult human, a dosage of 500 mg. of xanthopterin would probably be safe and this would provide a concentration of 70 microgram per ml. of blood, a concentration which should be adequate for the inhibition of tumors. It may be that such testing has already been done.

Purine, Pyrimidine, and Folic Acid Antagonists

It has been postulated that folic acid operates in the enzymatic synthesis of purines and pyrimidines (Frommeyer et al., 1946; Rogers and Shive, 1948; Spies, et al., 1946). Details of this enzymatic aspect are considered in the section on enzymes and folic acid analogues. The relationship of pyrimidine antagonists to folic acid is therefore of great interest. Hitchings et al. (1948) demonstrated reversal by folic acid of the growth inhibitory action of 2,6-diaminopurine and 2,4-diamino-6,7-dimethylpteridine for *Lactobacillus casei*.

The complexity of the problem of metabolite analogues and the difficulty of interpretation of experimental findings is evidenced in the maze of interrelationships of diaminopurines and pteridines with folic acid, with purines, and with pyrimidines. It is highly probable that no given metabolite antagonist functions with 100 per cent selectivity and this is the redeeming feature of the entire concept when it is applied to pharmacology and chemotherapy. If 100 per cent specificity did occur, differential requirements of parasite and host would be the sole determining factor for therapeutic index; however, as it is evident that each antagonist affects not one but several enzymatic systems, it becomes apparent that metabolite antagonists designed for a single metabolite may affect systems remote to that metabolite and by so doing bring into view pharmacological properties of great merit.

In the case of the sulfonamides we find interference not only with p-aminobenzoic acid systems and pteroylglutamic acid, but with secondary systems involving a diversity of metabolites such as methionine, purines, and others. Now pharmacological and chemotherapeutic power will be a direct function of the number of metabolic channels which are obstructed by a given molecule or series of molecules. As an example, if the ultimate goal is displacement of molecule A which is formed from B by enzyme C, then the logical approach is to design a displacer for metabolite A to block the reaction at its endpoint; to use an antagonist for molecule B to prevent the formation of A; and finally to obstruct the action of enzyme C by one or more of several procedures available via the metabolite displacement concept. The multiple approach becomes the valid and logical one; no single metabolite displacer could have the breadth of scope and the effectiveness of several such agents used in combination. This statement must be modified, viz., it is extremely improbable that a single molecule would be found possessing the merits of the combination. It is probable that one of the major pitfalls of the application of the metabolite displacer concept comes in an attempt to isolate a given reaction and its enzyme when we know that enzymes act in concert, that they perform in groups, that no single enzyme is an island unto itself; thus, our studies in isolated systems have validity only when viewed in the light of the complexity of the interrelated enzymatic processes which make up the anabolic and metabolic chains of living matter.

Animal Studies with Folic Acid Analogues

The first folic acid displacing agent to be synthesized and tested was methylfolic acid (Martin, et al., 1947) which has been variously designated as methylfolic acid, 7-methylfolic acid, x-methylfolic acid, and crude folic acid antagonist. The several designations are due to a divergence of opinion on the structure of the agent produced by the interaction of 2,4,5-triamino-

6-hydroxypyrimidine, p-aminobenzoylglutamic acid, and 2,3-dibromobutyraldehyde.

Franklin et al. (1947) confirmed the efficacy of this agent as a folic acid displacer for bacteria, and also tested it in rats. The antagonist was fed in the diet at a level of 1 to 10 grams per kilogram of diet. The syndrome produced was similar to but more acute than that induced by feeding purified diets with succinylsulfathiazole and included slowing of growth, reduction of hemoglobin, and marked reduction of the white count. The differential white count showed a more marked reduction in the granulocytes than in the lymphocytes. At autopsy, the oral cavity showed necrotic and ulcerative lesions. Reversal of the syndrome induced by the displacer was complete except that splenomegaly occurred and was not reversed. Irreversibility of antagonists is of tremendous importance in a consideration of possible clinical application. Any manifestation of the activity of a given molecule which is not reversible is a mark against that molecule from the standpoint of its ultimate toxicity on clinical application.

This same group of investigators (Franklin, et al., 1947a) subsequently extended their work to include mice and chicks. The deficiency states induced did not differ significantly from those seen in the rat. The authors emphasize the reversibility of the action of the antagonist. Welch et al. (1947) applied the same antagonist in the production of a pteroylglutamic acid deficiency in the pig. Their emphasis was on the fact that the effect of the methylfolic acid was reversed by the administration of a crude source of extrinsic factor (essentially free of PGA) together with normal human gastric juice. The technic was offered as a suitable bioassay tool for the study of antianemic substances.

The Cleveland group (Weir et al., 1948) also reported the production of folic acid deficiency in mice by the use of a folic acid antagonist. In the deficiency state, the marrow of mice showed maturation arrest with hyperplasia of immature elements and marked reduction of more adult forms. This led to the suggestion that pteroylglutamic acid was not necessary for the formation of the most immature blood elements but was required for maturation. Immature elements are generally seen in the peripheral blood of animals with antagonist-induced folic acid deficiency.

It is surprising that this antagonist, which is completely reversible, has not received clinical consideration of significance.

The focus of attention in the field of folic acid antagonists has been on 4-aminopteroylglutamic acid (aminopterin). The effect of this agent was studied in mice (Franklin, et al. 1948) and in rats and chicks (Oleson, et al., 1948). Death occurred in the test animal within a few days on levels of 1 part per million of diet. The action was not reversed even by feeding high levels of pteroylglutamic acid; this in direct contrast to bacterial test systems in which complete reversal occurred. This same antagonist was found ef-

fective in guinea pigs (Minnich and Moore, 1948) in which blood changes were induced which could not be reversed by either folic acid or liver extracts. If administration of the inhibitor was stopped, the hematological changes reversed and normality ensued. Simultaneous administration of folic acid and the antagonist resulted in prevention of the development of the leukopenia and thrombocytopenia but not of the anemia. 4-Aminopteroylglutamic acid was reported (Swendseid, et al., 1948) to be the most powerful inhibitor of folic acid in a series which included 2,4-diamino-6,7-diphenylpteridine, 4-desoxypteroylglutamic acid and 4-aminopteroylglutamic acid. The pteridine seemed to have a preferential effect on the leukocytes by contrast with the more complex molecules which affected both the red and white cells.

The St. Louis group (Innes et al., 1949) reported details of their earlier presentation of the hematological changes induced in guinea pigs by the administration of folic acid antagonists. Pteroylaspartic acid and N10-methylpterioic acid were included in this study, and were reported as producing little hematological effect when given alone but as possessing the capacity of potentiating the action of minimal dosages of 4-aminopteroylglutamic acid. Details of the bone marrow and peripheral blood hematological picture were presented.

The LD-50 of 4-aminopteroylglutamic acid is 1.9 mg. per kg. in mice and 4.5 in rats (Philips and Thiersch, 1949). Tenfold LD-50 dosages did not cause the animals to succumb any earlier than they did with a single LD-50, namely on the third day. Folic acid and pernicious anemia factor administration did not alter the acute toxicity of the compound. Severe, watery diarrhea characterized the state of toxicity. Rats receiving intraperitoneal doses of 1.25 mg. per kilogram of the antagonists survived 100 per cent; whereas doses as low as 0.25 mg. per kilogram given daily for eight days caused 60 per cent mortality. This is a point of major interest when one considers the potentiality of producing immediate profound deficiency states affecting rapidly metabolizing cancer cells but not normal tissue cells. The toxicity of these agents to cancer cells may approach immediacy, whereas the bone marrow effects take time to reach a climax. Microscopic pathology of animals treated with the antagonist show marked edema, desquamation of mucosa, and leukocytic infiltration. In general, the lymphoid tissues decreased in size. Additional histopathology of rats on 4-aminopteroylglutamic acid is presented by Higgins (1949).

Thiersch and Philips (1949) report the development in dogs under treatment with 4-aminopteroylglutamic acid of a sprue-like syndrome, characterized by diarrhea, peripheral leukopenia, depletion of bone marrow. The syndrome was essentially similar to that seen in rats, mice, guinea pigs, pigs, and monkeys. In the latter species, aminopterin induces creatinurea

during the induction of a deficiency state and during the recovery period (Dinning et al., 1949a).

Chick embryos are profoundly affected by folic acid antagonists. Wayley and Morgan (1949) report that 4-aminopteroylglutamic acid causes a marked diminution in the number and size of the blood islets in the yolk sac of seven day old embryos. Liver extract and vitamin B₁₂ did not reverse this effect but folic acid tended to reduce though not to eliminate the antagonistic action. N-Methylpteroic acid had no effect. Methyl-4-aminopteroylglutamic acid was less active. The 4-amino folic acid analogues with aspartic acid, threonine, and alanine replacing glutamic acid in the molecule were reported to be less toxic than 4-aminopteroylglutamic acid (Karnofsky et al., 1949) as tested. 2,6-Diaminopurine was not an active growth inhibitor of the chick embryo.

Ritter and Oleson (1949) presented one of the few reports indicating reversal of the toxic action of 4-aminopteroylglutamic acid by folic acid in the rat. Recently, 4-aminopteroylaspartic acid has been investigated in bacteria, in the chick and in the rat and found to produce essentially the same manifestations as does the corresponding glutamic acid molecule (Woll, 1949; Hutchings, et al., 1949). A difference existed in that the deficiency induced in the chick and the rat by 4-aminopteroylaspartic acid was not precipitous in nature but gradual in onset.

Animal studies have been conducted with one other folic acid antagonist, namely 4-amino-10-methylpteroylglutamic acid (Franklin, et al., 1949a). These studies demonstrated that the compound was an effective antagonist in the chick, rat, and bacterium. The action was completely reversible in the bacterial system but could not be effectively reversed in the rat. Oleson (1950) reports a series of new folic acid analogues which for purposes of brevity are included in Table III and not presented individually.

A notable observation concerns the failure of 4-aminopteroylglutamic acid to produce lymphopenia and acute atrophy of the lymphatic tissues following adrenalectomy (Dougherty and Dougherty, 1950). These investigators suggest that the action of the antagonists is at least in part hormonally mediated. The effect of the drug on myelopoiesis was not mediated even partially by the pituitary or adrenal cortex. Adrenal hypertrophy occurred following dosage with aminopterin and, conversely, the lymphocytosis and hypertrophy of the thymus characteristically observed after adrenalectomy was not found in the adrenalectomized aminopterin treated animals.

Animal Studies Involving Tumor Chemotherapy with Folic Acid Analogues

The first reports of the application of pteroylglutamic acid displacing agents in the chemotherapy of tumors appeared in 1948. Woll (1948)

studied the Rous chicken sarcoma and its reaction to folic acid, and derivatives, and to a folic acid free diet. Similar observations were reported by Little et al., (1948) in the same issue of the transactions of the New York Academy of Science.

It will be recalled that the Rous chicken sarcoma is a virus disease and that it does not necessarily bear any relationship to other types of tumors. Little and his associates were able to prevent tumor development in chicks maintained on a synthetic, folic acid free diet. It was therefore not surprising that the same result was obtained using folic acid displacing agents: 4-aminopteroylglutamic acid and 4-aminopteroylaspartic acid. It was suggested that the action of the antagonists is primarily that of prevention of the multiplication of the virus with consequent inhibition of growth of the sarcoma. The injection of 400 micrograms daily of 4-aminopteroylaspartic acid both decreased markedly the number of chicks developing tumors and caused a delay in the appearance of tumors in those chicks which were not completely protected. At doses of 100 micrograms daily, 4-aminopteroylaspartic acid and 4-aminopteroylglutamic acid with d(—)glutamic acid were far more active than 4-amino-N-methylfolic acid, 4-amino-N-methylpteronic acid, and pteroylaspartic acid. The folic acid antagonists were capable of preventing further growth of a tumor already established. An extension of nutritional studies on Rous chicken sarcoma indicated that tumor response was stimulated by folic acid, nicotinamide, calcium pantothenate, riboflavin, and cholic acid. Folic acid was however the only factor the absence of which completely prevented any tumor response (Little, et al., 1948a). These observations strongly suggest the probability that the use of displacement agents for several factors essential to the growth of the tumor might well result in more effective chemotherapy.

The Lederle group (Little, et al., 1948b), reporting extended studies, concluded: "It is evident from our experiments that Rous chicken sarcoma may be controlled by regulating the amount of pteroylglutamic acid in the tissues of the chicken, either by use of synthetic folic acid free diets or by use of suitable chemical antagonists." These investigators found 4-aminopteroylaspartic acid, 4-aminopteroyl-d(—)glutamic acid and 4-amino-N-methylpteroylglutamic acid effective agents for controlling available pteroylglutamic acid in the chick. With these compounds, it was possible to reach a differential point at which the body was provided with enough folic acid for growth while the tumor was deprived of adequate supplies of this factor. The toxicity of the chemicals was directly associated with the severity of the deficiency state produced. The dosage used for tumor inhibition in these experiments was 80 mg. per kilogram of diet which was fed ad libitum. The oral route of administration of the antagonists was recommended. The most extensive listing of analogues tested on Rous sarcoma is that presented by Oleson (1950) in Table IV.

Table IV†

THE INHIBITORY EFFECT OF 4-AMINO COMPOUNDS ON THE ACTIVITY OF
PTEROYLGLUTAMIC ACID IN GROWTH OF ROUS SARCOMA

Compound	Am't Inj. Daily/ Am't of PGA	Total No. of Chicks	% Chicks w. Tumors	Avg. Tumor Size
4-Aminopteroylglutamic acid (aminopterin)	10γ/10γ 10γ/250γ	0(10)* 6(10)*	— 83	— ++
4-Aminopteroylglutamyl glycine	10γ/10γ 25γ/10γ	2(10)* 0(10)*	0 —	0 —
4-Aminopteroylglutamyl-β- alanine	10γ/10γ 25γ/10γ	2(10)* 0(10)*	0 —	0 —
4-Aminopteroyl-d(—) glutamic acid	5γ/20γ 20γ/20γ	10 10	70 60	+++ +++
4-Aminopteroylaspartic acid	100γ/5γ 100γ/10γ 100γ/25γ	10 10 9	0 20 33	0 + +
4-Aminopteroylthreonine	10γ/10γ 25γ/10γ	10 10	50 20	++ +
4-Aminopteroyl-α-alanine	10γ/10γ 25γ/10γ	10 10	70 60	++ ++
4-Aminopteroylserylglutamic acid	50γ/20γ 100γ/20γ 200γ/20γ 500γ/20γ 1000γ/20γ	10 10 10 9 6	90 30 30 0 0	+++ ++ + 0 0

THE INHIBITORY EFFECT OF OTHER RELATED COMPOUNDS ON THE ACTIVITY OF
PTEROYLGLUTAMIC ACID IN GROWTH OF ROUS SARCOMA

9,10-Dimethylpteroylglutamic acid	10γ/10γ 100γ/10γ 500γ/10γ	10 9 10	20 22 30	++ + +
6-Methyl-7-pteroylglutamic acid	10γ/10γ 100γ/10γ 500γ/10γ 1000γ/20γ	10 9 8 10	50 77 87 80	++ ++ ++ +++
7-Methylpteroylglutamic acid	10γ/10γ 100γ/10γ 500γ/10γ	10 10 9	50 20 22	++ + +
7-Carboxy-6-pteroylglutamic acid	100γ/20γ 1000γ/20γ	9 9	100 88	+++ +++
Pteroyl-(d)-serylglutamic acid	50γ/20γ 10	7 20	28 85	++ ++
Controls	20	10	100	+++

† From Oleson, J. J., *Tr. N. Y. Acad. Sc.*, **12**, 118, 1950.

* Signifies the original numbers used where toxicity of compounds reduced the number of birds in the group.

It was to have been anticipated that other investigators would try this class of antagonists in different tumors. An early phase was that directed at hormone-induced changes in bodily structures. Hertz (1948), using a crude methylfolic acid preparation, demonstrated that this chemical markedly reduces the tissue growth response to maximally effective doses of diethylstilbestrol in the chick maintained on a normal stock diet. The inhibition was promptly reversed by folic acid supplements. The crude folic acid antagonist was fed at 1 per cent levels in the diet. Hertz indicates possible application of such observations to the control of those human cancers in which estrogens are known to play a role; thus, cancers of the prostate and breast are known to be retarded by partial elimination from the body of the hormones involved in the normal metabolism of these organs. Inhibition of oviduct response to estrogens in frogs had been shown to be markedly decreased (Goldsmith et al., 1948-49) by the administration of 4-aminopteroylglutamic acid. The effect was not reversed by concentration of folic acid exceeding that of the antagonists by a factor of 100. The list of antagonists effective in the estrogen-induced tissue response system was enlarged (Hertz and Tullner, 1949) to include 4-amino-N10-methylpteroylglutamic acid, 4-aminopteroylaspartic acid, and 4-desoxypteroylglutamic acid. A series of four pteridines proved ineffective in the dosages employed. These studies rather clearly demonstrate that any folic acid antagonist will possess the capacity to inhibit hormonally induced changes in various organs. Biochemical evidence of the efficacy of folic acid antagonists in the prevention of estrogen response lies in the decreased amount of alkaline phosphatase in the oviduct of the chick after estrogen treatment (Andrus and Zarrow, 1949).

Brendler (1949) reasoned that if folic acid antagonists inhibited estrogen-induced responses in tissues they should also nullify the depressant effect of estrogens on the rat prostate. His work demonstrated that this was in fact the case. 4-Aminopteroylglutamic acid interfered with the ordinary depressive influence of alpha-estradiol on the rat prostate. The antagonist itself possessed no androgenic potential nor did it interfere with the androgen stimulation of tissue growth, thus the average weight of rat prostate following testosterone was 380 mg. and following testosterone and 4-aminopteroylglutamic acid it was 406 mg. This is rather startling because the implication is that the antagonist interferes with positive and negative actions of the estrogens and does not interfere with the positive action of an androgen. The problem is the more confusing because Goldsmith et al. (1949a; 1949b) have reported that the seminal vesicles of castrated male mice responded only slightly to high dosages of testosterone if pretreatment with crude methylfolic acid had taken place. 4-Amino-N10-methylpteroylglutamic acid gave the same result as was seen with the crude methylfolic acid.

It would be phenomenal if this sex hormone response as related to folic acid differed between the androgenic and estrogenic agents. It seems probable that if this were the case the observation would be the first of its kind.

Mouse leukemia has also responded to metabolite antagonists of this class. Thus, the transplanted leukemia of Ak mice is partially controlled either by a folic acid free diet or by a crude methylfolic acid compound (Weir et al., 1949). Parallel observations (Burchenal et al., 1949, Law et al., 1949; Burchenal et al., 1949a) have established the effectiveness of 4-amino folic acid antagonists against some strains of transplanted mouse leukemia. The prevention of the effect of 4-amino-N10-methylpteroylglutamic acid on mouse leukemia by folic acid has been demonstrated by Burchenal et al. (1949b). Ten to 20 times as much pteroylglutamic acid as antagonist was required for nullification of the displacer action. The induction of resistance to 4-amino-N10-methylpteroylglutamic acid in a strain of transmitted mouse leukemia has been reported (Burchenal et al., 1950) and offered as a possible explanation of the eventual failure of folic acid antagonist therapy in patients with acute leukemia.

Sarcoma 180 is inhibited in its development of 4-amino-N10-methylpteroylglutamic acid (Moore et al., 1949). The compound is stated to exhibit the most favorable therapeutic index of some 700 miscellaneous compounds tested. It was administered intraperitoneally in concentration of 1.5 mg. per kilogram per day for seven days. Few mice died on this level. The inhibition was most apparent when the compound was administered early. The corresponding compound (Sugiura et al., 1949), 4-aminopteroylglutamic acid, was also effective against sarcoma R 39 in rats. The therapeutic index was poor and many treated animals died. The compound at toxic levels inhibited the growth of sarcoma 180, mammary adenocarcinoma EO 771, and Harding Passey melanoma in mice. It was inactive against spontaneous breast cancer in mice and did not affect the growth capacity of tumor fragment of sarcoma 180, adenocarcinoma EO 771, or Harding Passey mouse melanoma when these tumors were exposed for 24 hours to solutions containing 0.1 per cent 4-aminopteroylglutamic acid. Neither 4-aminopteroylglutamic acid nor 4-amino-N10-methylpteroylglutamic acid produced permanent damage to erythrocyte production mechanisms or to sarcoma 180 (Thiersch and Stock, 1949), complete recovery occurring within five days after cessation of treatment with the metabolite antagonists. Schoenbach et al. (1949) reported that daily doses of 0.2 to 0.3 mg. per kilogram of 4-aminopteroylglutamic acid administered to mice diminished the weight of tumors (sarcoma 180) and caused a marked alteration in histological appearance. The lethal dose of the metabolite antagonist was 1.2 to 1.4 mg. per kilogram in mice. Complete reversal of the 4-aminopteroylglutamic acid inhibition of sarcoma 180 is effected by folic acid (Goldin, et al., 1949).

The most recent application of folic acid antagonists has been in the field of plant tumors. DeRopp (1949) used carrot fragments on which tumors had been induced by crown gall bacteria as testing systems. 4-Aminopteroylglutamic acid, 4-amino-N10-methyl-PGA, 4-amino-9,N10-dimethyl PGA, and 4-amino-9-methyl-PGA were all highly effective inhibitors. Pteroylaspartic acid, pteroyl-dl-aspartic acid, N10-methylpteroic acid, 9-methyl-PGA, 4-aminopteroylaspartic acid, 4-aminopteroyl-dl-2-alanine, and 4-amino-dl-threonine were all inactive or relatively so.

Thus, it is apparent that folic acid antagonists are effective inhibitors of the Rous chicken sarcoma, mouse leukemia, sarcoma 180 of mice, mammary adenocarcinoma, and Harding Passey melanoma in mice. Many different types of metabolite antagonists possess cancer inhibitory capacity, and not more than a dozen different structural modifications have been tested out of a possible hundred or more such molecules. With the evidence at hand, it is vital that all structural analogues of pteroylglutamic acid possessing activity as antagonists be tried in tumor chemotherapy, alone and in combination with other metabolite displacers.

The time factor must be given consideration, i.e., it may be possible to produce for a limited period of time a marked folic deficiency which will result in the death of the more rapidly metabolizing tumor cell without serious injury to the tissue body cell. Considering the vast possibilities, work in this field has only begun.

Clinical Studies Involving Metabolite Analogues of Folic Acid

The clinical application of metabolite analogues of pteroylglutamic acid was stimulated by earlier reports on the use of pteroyltriglutamic acid (teropterin). Farber and his associates (1947) treated some 90 patients with malignant disease using teropterin and diopterin. The patients were far advanced cases with acute leukemia, carcinoma, Hodgkin's disease, osteogenic sarcoma, and many other types of malignancies. Teropterin was used in dosages of from 10 to 150 mg. intramuscularly and from 20 to 500 mg. intravenously. The conclusion was reached that the drugs were nontoxic and that improvement in energy, appetite, and sense of well-being occurred. However, an analysis of all clinical findings with teropterin is presented by Rhoads (1949) and it would seem that despite the few favorable reports teropterin can be dropped from consideration in the chemotherapy of cancer. Its use, however, was an important factor in the growth of interest among clinicians in the true antagonists of folic acid.

The relationship of folic acid to the bone marrow and to the blood forming system in general made obvious the probability that folic acid antagonists would be effective in the treatment of the leukemias. Acting on this probability, Farber et al. (1948) tested the activity of 4-aminopteroylglutamic acid on leukemia in children. The compound was found to be

toxic but it did induce temporary remissions. The use of pteroylaspartic acid on 14 children with leukemia and of methylpterotic acid on seven similar cases is recorded in this report. One case treated with pteroylaspartic acid in doses of 40 mg. showed at autopsy a markedly hypoplastic bone marrow. Farber dismissed both pteroylaspartic acid and methylpterotic acid with the comment that the results were encouraging and led to the testing of aminopterin. It is interesting that 4-aminopteroylglutamic acid was selected because it was well known that the toxic effect of this compound cannot be reversed in mice by the administration of folic acid. Of the 16 patients treated with aminopterin, ten showed clinical, hematological, and pathological evidences of improvement of important nature for a period of three months, which was the duration of the experiment at the time it was reported. The average dosage was 1 mg. of aminopterin given daily by the intramuscular route. Under treatment with aminopterin, the white cell count returned to normal whether it was initially high or low. The peripheral blood changes indicated an approach to the normal with respect to hemoglobin, red cell count, and platelets. Farber mentions the toxic manifestations as being primarily stomatitis with early ulceration. One is led to wonder if ascorbic acid metabolism might not be involved in the toxic lesions of the oral cavity, particularly in view of the work of Johnson and Dana (1948) demonstrating ascorbic acid as a complicating factor in folic acid deficiency in the rat.

Spies and his associates (1947) first tested metabolite analogues of folic acid in human beings. They were interested in checking the specificity of the folic acid molecule and reported that methylfolic acid was ineffective in stimulating blood regeneration in two persons with macrocytic anemia. Methylfolic acid was administered in doses of 20 to 40 mg. orally daily for 12 days. Later these investigators tried methylfolic acid, N-(4-((4-quinazoline)amino)benzoyl)-glutamic acid, the magnesium salt of formylpteroylglutamic acid, the magnesium salt of formylpterotic acid, pteroylaspartic acid, oxyfolic acid, and oxypterotic acid (Spies, et al., 1948, Scholz et al., 1948). In the amounts administered, only the magnesium salt of formylpteroylglutamic acid effected a reticulocytosis and an increase in red blood cells.

One most interesting phase of the specificity aspect of folic acid has been disclosed by the work of Meyer et al., (1949) who found that 5 mg. of 4-amino-10-methylpteroylglutamic acid prevented the reticulocyte response which normally follows the administration of 10–15 gamma of vitamin B₁₂ in the pernicious anemia patient in relapse. This seems to indicate that B₁₂ will not act in the absence of folic acid and that the two should be used together in the treatment of pernicious anemia.

Meyer (1948) used N-(4-((2-amino-4-hydroxy-6-pteridyl)methyl)-amino)benzoyl)-d,l-aspartic acid, and 4-(N-((2-amino-4-hydroxy-6-pteridyl)methyl)-amino)benzoyl)-d,l-aspartic acid, and 4-(N-((2-amino-4-hydroxy-6-pteridyl)methyl)-amino)benzoyl)-d,l-aspartic acid.

dyl)methyl)N-methylamino)benzoic acid in leukemic patients and found that there was a reduction in the total white cells with a temporary shift to the right. Dosages ranged from 20 to 80 mg. daily intramuscularly.

In 1948, two reports appeared both confirming and extending the original observations on the efficacy and limitations of folic acid antagonists in leukemias. One of these (Pierce and Alt, 1948) records results using aminopterin in 11 cases of acute leukemia. Remissions were obtained in five instances. Dosage was 0.25 to 1.0 mg. daily in children and from 1 to 2 mg. in adults. Berman et al., (1948) emphasized the toxic effects produced by aminopterin. These include: pharyngitis, stomatitis, sore tongue, crampy abdominal pain, diarrhea, dermatitis, and alopecia. Pharyngitis appeared almost simultaneously with improvement in the white count. The patients in their series were all chronic leukemics and the overall picture indicates that toxic manifestations supervene before clearcut improvement occurs.

In 1949, further work established the merit of this approach to the problem of acute leukemia. Dameshek (1949) used a series of folic acid antagonists and reported that one third of acute and subacute leukemic patients whether adults or children improved under treatment. Results were best with relatively subacute cases of lymphoblastic or myeloblastic type disease. The effect was only temporary and death occurred. Toxicity reactions were as reported by others. Tumor patients treated with folic acid antagonists show a reduction in the number of myeloid and red blood cells in the bone marrow (Thiersch, 1949). Morphological changes in the circulating red blood cells also occur in about 30 per cent of patients treated with aminopterin. Both macrocytosis and anisocytosis are noted. The series of acute leukemics treated by Stickney et al., (1949) included 21 children and 33 adults. The plan of treatment involved the use of 1 mg. of aminopterin intramuscularly or orally every day until toxic manifestations appeared. The discouraging aspect was that the improvements observed could not be maintained. Complete remissions lasting up to four months were achieved in five of the children and three of the adults. No remissions were maintained indefinitely.

In 1950, the final phases of determination of limitation and application of the folic acid antagonists have been reported. Thus, Ross and Schoemperlen (1950) reported a single case in which aminopterin controlled the acute stem cell leukemia. The absolute number of stem cells was reduced from 18,000 per cu. mm. to 16 per cu. mm. Toxic effects and a failure to maintain the remission were noted. A series of 24 cases of acute leukemia in children under treatment with aminopterin and other folic acid antagonists was recorded by Weber et al. (1950) who noted hematological and symptomatic improvement, or both, in 20 of the cases. They used daily dosages of 1 mg. of aminopterin intramuscularly. It seems clear from this report and others that the first manifestation of toxicity is ulcerative lesions

of the oral mucous membranes. This group of investigators conclude that while the antagonists did not constitute a satisfactory form of therapy, they were more effective than other forms of treatment. Sachs et al. (1950) report 14 cases of acute leukemia; four were myeloid, seven lymphoid, and three acute monocytic in type. Two patients showed complete temporary clinical and hematological remissions, three showed partial temporary remissions, and the course of the disease was uninfluenced in nine patients. The duration of remission in one patient was 81 days followed by relapse and death. The series of leukemics studied by Fowler (1950) show essentially the same statistical results.

The use of folic acid antagonists is one of the most striking inroads yet made into the realm of the chemotherapy of cancer. It is not a solution to any phase of the problem as it now stands but considering the hundreds of possible metabolite antagonists of folic acid it is immediately apparent that many potentialities remain. The toxicity need not necessarily be associated with the therapeutic efficacy. This aspect can readily be studied in animals. The remissions are the main problem. In this, it seems not improbable that the use of multiple agents inhibiting more than one metabolic channel might prove of value. There are so many metabolic channels in any system that blocking a single one would hardly form the best approach. Folic acid antagonists used in conjunction with purine displacers should be tried; many probabilities immediately present themselves as requiring attention and resolution.

Recapitulation

Effective metabolite analogues for pteroylglutamic acid have been created by modification in each of the three major subdivisions of the molecule. Thus, 4-aminopteroylglutamic acid represents a pteridyl moiety modification; pteridylsulfoglutamic acid a p-aminobenzoic acid moiety modification; and pteroylaspartic acid a glutamic acid moiety modification. Displacers in this series are known with action inhibitory to the growth of bacteria requiring preformed folic acid and to that of bacteria which can synthesize this vitamin. Metabolite analogues can prevent the formation of the factor and interfere with its function as a prosthetic grouping or as a cofactor in enzymatic processes. To date, there is no recorded instance of inhibition of the action of a preformed pteroylglutamic acid enzyme.

Important developments have resulted from study of pteridine molecules as folic acid metabolite analogues. Pterines have been found to act as displacers for preformed folic acid and to inhibit its formation. Furthermore, purine analogues such as 2,6-diaminopurine are effective displacers. The demonstrated synergism of sulfonamides and pteridines against *Staphylococcus aureus* and *Escherichia coli* strongly invites extension of studies to combinations of metabolite analogues throughout the entire field.

The syndrome produced by synthetic folic acid free diets and that precipitated by metabolite analogues of the vitamin is identical. One manifestation of the deficiency state produced in animals was a marked lowering of the white count, which observation led to the application of folic acid displacers in the chemotherapy of the leukemias. The limited efficacy of this approach to the problem of the control of malignancy has been most encouraging. Development of resistance by a strain of transmitted mouse leukemia to the chemotherapeutic action of metabolite analogues of pteroylglutamic acid is offered as the explanation of the eventual failure of this type therapy in patients with acute leukemia. In any event, the displacement concept has afforded the most hopeful approach to the problem of malignancy, and extension of study along these lines may well bring a resolution.

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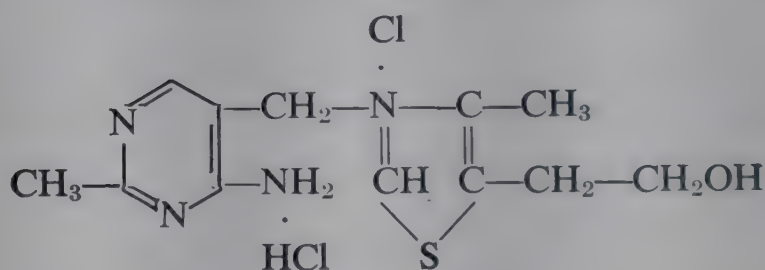
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Chapter 13

METABOLITE ANALOGUES OF THIAMINE AND RIBOFLAVIN

Metabolite Analogues of Thiamine



Thiamine

4-methyl-5- β -hydroxyethyl-N-((2-methyl-4-aminopyrimidyl-(5))-methyl)-thiazolium-chloride-hydrochloride

1. Metabolite Analogues with Thiazole Moiety Modification.
2. Metabolite Analogues with Pyrimidine Moiety Modification.
3. Enzymes and Metabolite Analogues of Thiamine.
4. Thiaminase.
5. Recapitulation.

Thiamine is an essential factor for plants, microorganisms, and animals. In the animal classification, all species require the vitamin preformed. Microorganisms can be classified into two general categories; those which can synthesize the nutrilit and those which cannot. Further subdivisions can be made into those which are capable of synthesizing thiamine from the pyrimidine and thiazole intermediates; those which are able to synthesize only one part of the molecule, either the pyrimidine or the thiazole moieties, and therefore require the other segment preformed for coupling, and finally those which can synthesize both portions of the molecule and subsequently synthesize the factor.

Thiamine is active as the pyrophosphoric acid ester which is a specific coenzyme for the carboxylase in the oxidation of pyruvic acid. Animal tissues contain the vitamin largely in the form of the ester cocarboxylase. It is probable that the biochemical defect of thiamine deficiency states is primarily that of the absence of cocarboxylase which results in pathological

states such as peripheral neuritis, bradycardia, hypomotility, and atony of the gastrointestinal tract and similar symptoms.

Enzymatic destruction of thiamine by raw fish meal results in some animals in the development of a deficiency disease known as Chastek paralysis. Clams also contain thiaminase.

Chemically, thiamine consists of the thiazole and a pyrimidine moiety linked through the methylene bridge. Either major segment of the structure might be modified in whole or in part for the production of metabolite analogues. The methylene bridge might well be altered with consequent production of displacers.

Metabolite Analogues with Thiazole Moiety Modification

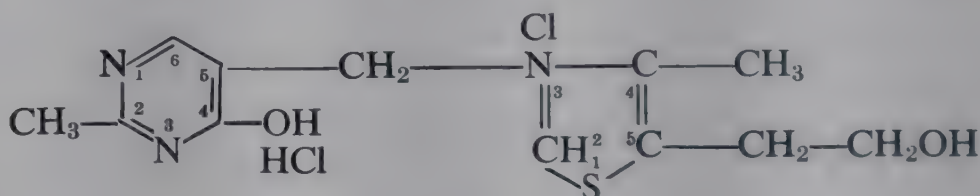
Entry into this phase of the displacement problem is credited to Woolley (1943), although Robbins (1941) had reported that the pyridine analogue of thiamine inhibited the growth of certain fungi. Woolley and White synthesized the pyridine analogue of thiamine, (2-methyl-4-amino-5-pyrimidylmethyl-(2-methyl-3-hydroxyethyl) pyridinium bromide) (Fig. 1), and found that this analogue produced in mice a characteristic deficiency which could be either prevented or cured by feeding sufficient amounts of thiamine. These authors calculated that it took about 40 molecules of pyrithiamine to nullify the effect of 1 molecule of thiamine. This fact makes pyrithiamine one of the most powerful vitamin antagonists yet known.

Many bacteria were found susceptible to the thiamine displacing action of pyrithiamine (Woolley and White, 1943a) and as would be anticipated the susceptible species were those requiring added thiamine for growth, while those not requiring added thiamine were not susceptible. Organisms grown in media containing pyrithiamine did not produce increased amounts of thiamine, and it is suggested by Woolley and White (1943a) that the nonsusceptible organisms do not owe this characteristic necessarily to thiamine synthesis. In keeping with the general plan, organisms requiring intact thiamine were about 10 times as sensitive to pyrithiamine as those requiring some portion of the thiamine molecule, such as the pyrimidine portion. The ratio was 100 times for those bacteria requiring both the pyrimidine and thiazole halves of the vitamin. Using a strain of *Endomyces vernalis*, Woolley (1944) developed a type which was resistant to the action of pyrithiamine. This resistant type still required thiamine or its pyrimidine portion as a growth factor. Pyrithiamine functioned as a growth factor in the absence of thiamine, and was reported to have approximately 50 per cent of the activity of the vitamin.

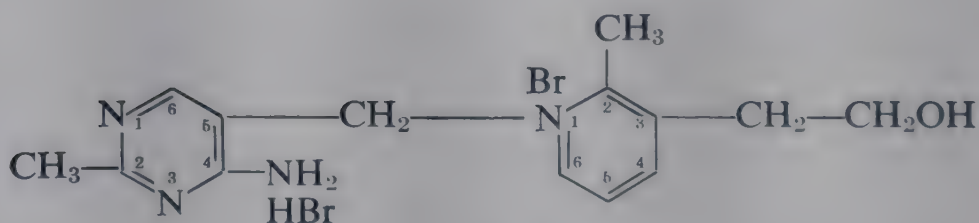
Pyrithiamine was studied in its effect on the growth promotion by thiamine of *Phycomyces blakesleanus* and *Ustilago violacea* by Schopfer and Boss (1948). With both microorganisms, pyrithiamine proved to be an effective inhibitor: the antagonism could be completely overcome by increased

amounts of thiamine. As a source of pyrimidine for *Boquet* strain of *Mycobacterium paratuberculosis* (Lutz, 1948), pyrithiamine was not effective. Other strains, listed as the *Pellegrini* and *Sohn* variety, utilized pyrithiamine completely as a source of pyrimidine. Of the varieties tested, the *Boquet* strain was the only acid fast type.

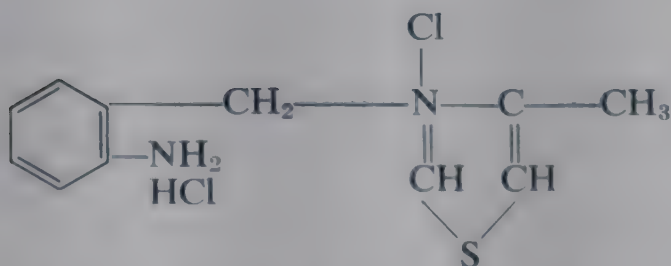
In June of 1949, Wilson and Harris reported that the method of Tracy and Elderfield (1941) which had been used by Woolley and White (1943) to synthesize the chemical which was designated as pyrithiamine actually produced a mixture of compounds of unknown structure. The name, neopyrithiamine, was used to designate the compound synthesized by these investigators and established to possess the structure which had originally been assigned to pyrithiamine. Pyrithiamine is probably a mixture of compounds having the pyridine and pyrimidine moieties present in ratios of 1:2.



Oxythiamine
4-methyl-5- β -hydroxyethyl-N-
((2-methyl-4-hydroxypyrimidyl-(5))-methyl)-
thiazolium-chloride-hydrochloride



Neopyrithiamine
1-((4-amino-2-methyl)-5-pyrimidylmethyl)-
2-methyl-3-(β -hydroxyethyl)-pyridinium-
bromide-hydrobromide



3-(o-aminobenzyl)-4-methylthiazolium-
chloride-hydrochloride

FIG. 1. Metabolite analogues of thiamine.

1:3, 1:4, etc. The biological activity of neopyrithiamine is reported as being four times that of pyrithiamine, the index of inhibition being about 10:1.

The finding that the structure originally assigned to pyrithiamine does not actually apply hardly invalidates the biological studies on this compound. It does, however, confuse the issue, in that it may be that only one molecular species in the mixture called pyrithiamine actually possesses the anti-vitamin action. There may be, therefore, an exceptionally powerful antagonist present or a series of antagonists weaker than neopyrithiamine.

Robbins (1942) reported studies with still another type of thiamine analogue, the pyrimidine analogue. It was neither beneficial nor detrimental to the growth of *Phycomyces*. The conclusion to be drawn is that in the systems tested this molecule did not act as a metabolite displacer of thiamine. This is rather strange, inasmuch as Robbins (1941) had noted growth inhibition of fungi by pyrithiamine.

Metabolite Analogues with Pyrimidine Moiety Modification

In 1944, Soodak and Cerecedo studied oxythiamine which had been prepared by Bergel and Todd (1937) and by Slobodin and Ziegel (1941). Bergel and Todd had reported absence of antineuritic properties of oxythiamine at 1 mg. doses when assayed by the bradycardia method. Soodak and Cerecedo prepared oxythiamine by the deamination of thiamine using nitrous acid. It produced a toxic effect in mice. Thus administration of the chemical at levels of 25 to 50 micrograms per day produced death in two weeks in young mice on a thiamine low diet supplemented by 1 microgram of thiamine.

Direct evidence of the effectiveness of oxythiamine as a metabolite displacer for thiamine has been presented by Frohman and Day (1949). They found that oxythiamine produced an accumulation of pyruvic and lactic acids in the blood of rats. Reversal of the effect was produced by thiamine. Quantitative data indicated that 50 gamma of the analogue produced symptoms of acute thiamine deficiency when the animals were receiving approximately 24 gamma of thiamine per day. The data of these investigators indicate that oxythiamine is a more powerful antivitamin than is either pyrithiamine or the 2-n-butylpyrimidine analogue of thiamine. In the chick, oxythiamine is an effective metabolite analogue (Daniel and Norris, 1949).

A direct comparison of oxythiamine and neopyrithiamine has been made by Eusebi and Cerecedo (1949) with neopyrithiamine found to be the more powerful antagonist. In this instance a ratio of thiamine to an antagonist of 1:50 was used. Strikingly enough, oxythiamine produced no polyneuritis but the animals died in 13 to 21 days. Neopyrithiamine produced a 100 per cent incidence of polyneuritis within seven days, and the survival period was from seven to eight days. The absence of polyneuritis in the series receiving oxythiamine established the basic differences which can exist be-

tween displacers aimed at the same target. The replacement of 4-position amino group of the pyrimidine by a hydroxy group produced a displacer causing death with no polyneuritic symptoms, while replacing the thiazole nucleus by a pyridine ring causes a 100 per cent incidence of polyneuritis. This must be a clue to the specificity of segments of the thiamine molecule in different physiological and biochemical functions normally performed by the entire molecular unit. The chemistry of oxythiamine has recently been presented (Soodak and Cerecedo, 1949). It might be noted in conclusion that bromoxythiamine is not an effective metabolite analogue (Eusebi and Cerecedo, 1950).

Another derivative of thiamine, the 2-n-butylpyrimidine homologue has been found (Emerson and Southwick, 1945) to produce thiamine deficiency in rats. The effect of 40 moles of the homologue was nullified by 1 mole of thiamine. The corresponding 2-ethyl derivative possessed full thiamine activity when tested with rats (Stein et al., 1941), and the 2-n-propyl analogue was active in pigeons (Schultz, 1940). It is surprising that such slight modifications in inert and theoretically nonfunctional groups such as alkyl radicals result in complete reversal of vitamin function. It would be interesting and theoretically of great importance to see if side chains of greater dimensional deviation, such as pentyl, hexyl, etc., would possess antivitamin properties.

Potentialities of the application of thiamine displacing agents in the field of chemotherapy became apparent with the publication of the work of Jones et al. (1948). Using the *Lansing* strain of poliomyelitis virus in white Swiss mice, they found that oxythiamine gave a significant degree of protection. The protection afforded was not quite as marked as that seen in mice on a low thiamine diet (Foster et al., 1942). Results indicate protection during the earlier phases of the experiment; that is, up to about the 21st or 22nd day there is a marked protective action which begins then to fade out. Biological time seems to play a role; it has been proposed to try acute and marked deficiency states produced by oxythiamine, the thought being that there would be a differential time element in susceptibility of host cell and virus to an acute and extreme deficiency state induced by oxythiamine.

Enzymes and Metabolite Analogues of Thiamine

Sarett and Cheldelin (1944) found that less pyriethiamine was required to inhibit the growth of *Lactobacillus fermentum* and *Penicillium digitatum* when diphosphothiamine (cocarboxylase) was used as growth factor than when thiamine was used. They offer as an explanation of this observation the suggestion that thiamine must be attached to the carboxylase protein before it is phosphorylated, thus forming a carboxylase enzyme different from that obtained when diphosphothiamine is supplied. 6-Aminopyrimidine compounds also inhibited the utilization of diphosphothiamine. This

effect was not characteristic of 6-oxypyrimidines such as uracil, which is 2,6-dioxypyrimidine. Two 6-aminopyrimidines were tested: 2-methyl-5-ethoxymethyl-6-aminopyrimidine and 2-methyl-6-aminopyrimidine.

Further potentiality for enzymatic studies with thiamine analogues comes from the work of Silverman (1949), who reports the inhibition of the synthesis of thiamine by atabrine in growing cells of *Torula utilis*. The observation of Silverman that atabrine competitively inhibits the dephosphorylation of cocarboxylase by a dried yeast preparation is interesting, viewed in the light of the results of Sarett and Cheldelin (1944), showing that pyrithiamine was more effective against cocarboxylase than against thiamine itself. It may well be that inhibition of the dephosphorylation of cocarboxylase forms the basis of the action of pyrithiamine in antagonizing the growth promoting action of cocarboxylase on *Lactobacillus fermentum*, this despite Silverman's statement to the effect that the atabrine inhibition of cocarboxylase dephosphorylation had nothing to do with the growth inhibitory effects of the drug. His conclusion was based upon the failure of cocarboxylase to accumulate in cells grown in the presence of atabrine.

Eusebi and Cerecedo (1950) have largely clarified the picture of enzymatic mechanisms and metabolite analogues of thiamine. While oxythiamine monophosphate and neopyrithiamine were inactive, oxythiamine diphosphate (262 γ) produced a 77 per cent inhibition of pyruvate decarboxylation in the presence of 4 γ of cocarboxylase. Oxythiamine on the other hand prevented the formation of cocarboxylase.

Thiaminase

Sealock et al. (1944) have made an interesting approach to the general problem in their use of various molecules in an attempt to inhibit the action of the enzyme in fish tissue which destroys thiamine. 3-o-Aminobenzyl-4-methylthiazolium chloride (Fig. 1) was active and the mechanism suggested competitive displacement. o-, m-, and p-Aminobenzyl derivatives of 2- or 4-methyl- and 2,4-dimethylthiazolium chlorides were tested. The results disclosed that the amino group was not only essential to the inhibition but that it must occupy the ortho position, the meta and para compounds being ineffective. Further, the unsubstituted benzyl-4-methylthiazolium chloride was inactive. The methyl group in the 2 position produced an effective agent, provided the o-aminobenzyl radical was present. Either the 2- or the 4-position must be unsubstituted for this action, as the 2,4-disubstituted molecule was ineffective.

Subsequently from this same laboratory (Livermore and Sealock, 1947) came a report of the synthesis of additional benzyl-(3)-thiazolium chloride analogues of thiamine. Included were the benzyl, o-, m-, or p-nitrobenzyl and the o-, m-, and p-aminobenzyl quaternary salts of 2- or 4-methyl, 2,4-dimethyl, and 4-methyl-5- β -hydroxyethylthiazoles. Their earlier report

(Sealock et al., 1944) had indicated that the hydroxyethyl side chain was not a prerequisite for activity.

In 1948, Sealock and Livermore confirmed their earlier findings on the effectiveness of *o*-aminobenzyl-(3)-4-methylthiazolium chloride as an inhibitor of thiamine destruction by fish tissue enzymes. They reported that the degree of inhibition was increased by the inclusion of extra manganous ion. The *m*-aminobenzylthiazolium salts not only failed to inhibit the enzymatic destruction of thiamine but actually caused increased destruction or activation. The increased destruction of thiamine occurred only in the presence of the enzyme extracts and not when thiamine was incubated with the analogue alone.

Oxythiamine, typical of metabolite analogues of thiamine with pyrimidine moiety modification, is an effective inhibitor of the action of thiaminase on the vitamin (Soodak and Cerecedo, 1944). On the other hand, neopyrithiamine while it inhibits destruction of thiamine by the enzyme, does so because it is in itself a substrate and is destroyed by thiaminase (Sealock and White, 1949).

Recapitulation

Thus, in summary, it is apparent that the relatively small amount of work done in this field is associated with the difficulty of synthesis of thiamine analogues. There are but four certain metabolite analogues of thiamine which have been properly identified, and these have been investigated biologically in only a restricted sense. The material originally designated as pyrithiamine is of unknown composition and since the same method was used in the synthesis of the pyrimidine analogue used by Robbins (1942), it is probable that this molecule is not currently identifiable. Thus, we find from our limited knowledge that metabolite analogues of thiamine can be prepared by replacing the thiazole nucleus by pyridine, by replacing the 4-amino group of the pyrimidine moiety by an hydroxyl, and by replacing the 2-methyl radical of the pyrimidine moiety by an *n*-propyl unit. The compound—3-(*o*-aminobenzyl)-4-methylthiazolium chloride—represents a special case tested only in the fish enzyme system. It may or may not be a true displacing agent.

As with other B-complex factors, there are metabolite analogues of thiamine which (pyrithiamine) prevent the synthesis of this factor; which (oxythiamine) prevent the incorporation of the molecule into a coenzyme; and which (oxythiamine diphosphate) prevent the action of the formed cocarboxylase. This pattern represents a general rule which can be applied to all B-complex factors.

The inadequate study of the metabolite analogues of thiamine is apparent. Dozens of additional analogues are known but have never been tested as thiamine displacers. One striking feature observed in the study of

this series is the failure of oxythiamine to produce polyneuritis in contrast to the 100 per cent incidence of this manifestation in pyriithiamine treated animals. This observation establishes the individuality of metabolite analogues as reflected in biochemical and physiological manifestations of this toxicity.

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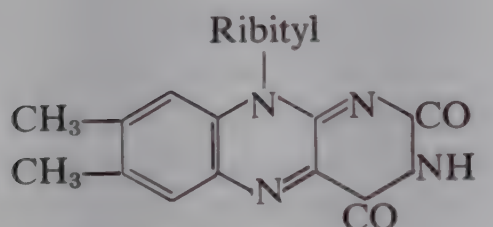
activity. As a model for the production of metabolite analogues, riboflavin clearly offers potentialities through change of sugar moiety, modification of the 6,7-dimethyl units, or change in ring structure.

General Studies of Analogues in Microorganisms and in Animals
Metabolite Analogues with 6,7-Dimethyl Modification

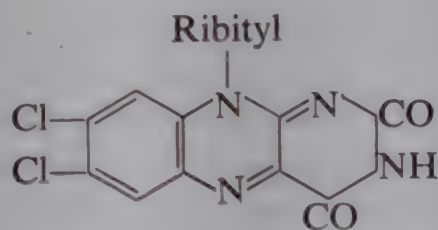
The first of the riboflavin antagonists to be designed (Kuhn, Weygand, and Moller, 1943), was 6,7-dichloro-9-d-riboflavin (Fig. 2), which completely inhibited the growth of *Staphylococcus aureus* in concentration of $1-6 \times 10^{-5}$ grams per cc. Substitution of methyl by chloro in the 6- or 7- or 6,7 positions was most effective.

Further investigation of the 6,7-dichloro analogue was reported by Schopfer (1948) who noted inhibition of the growth of *Eremothecium ashbyii*, a microorganism which is autotrophic and capable of producing riboflavin. Schopfer concluded that the inhibition was not due to displacement; however, this is not a valid position since the analogue may have been preventing the formation of riboflavin.

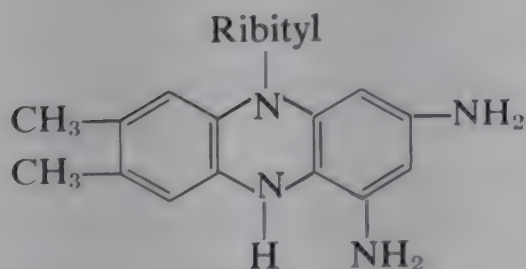
Emerson and Tishler (1944) reported the production of a riboflavin deficiency by feeding isoriboflavin which is the 5,6-dimethyl analogue (Fig. 2) to rats on diets containing suboptimal amount of riboflavin. The antago-



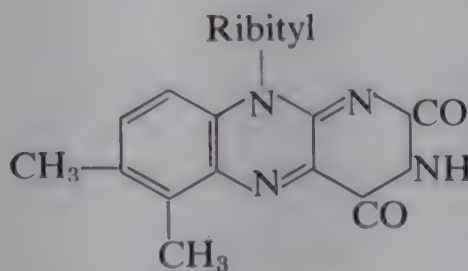
Riboflavin
6,7-dimethyl-9-(d-l'-ribityl)-
isoalloxazine
6,7-Dimethyl-9-d-riboflavin



6,7-Dichloro-9-d-riboflavin



2,4-Diamino-7,8-dimethyl-
10-ribityl-5,10-dihydrophenazine



Isoriboflavin
5,6-dimethyl-9-(d-l'-
ribityl)-isoalloxazine

FIG. 2. Riboflavin and displacers.

nistic effect of this isomer was prevented by the feeding of adequate levels of riboflavin. Isoriboflavin was not oxidized by the riboflavin-oxidizing enzyme of *Pseudomonas riboflaviano* and had less than 0.5 per cent of the activity of riboflavin for *Lactobacillus casei*; it had no effect upon acid production by this organism in the presence of maximal amounts of riboflavin (Foster, 1944). Sarett (1946) observed that in the presence of sub-optimal levels of riboflavin or of flavin-adenine-dinucleotide, isoriboflavin stimulated acid production by *Lactobacillus casei*. This is interpreted as stimulation of the utilization of riboflavin and flavin-adenine-dinucleotide by the bacteria.

Isoriboflavin is therefore an effective metabolite antagonist for riboflavin in the rat but is not effective in the bacterial systems studied. The diaminophenazine analogue is effective in both the rat and bacteria. This variation in capacity of metabolite analogues to function as antagonists depending upon the test mechanism is common to the field in general.

Metabolite Analogues with 9-Sugar Moiety Modification

Several derivatives of riboflavin were made by Kuhn et al. (1943) in which the substituent in the 9-position was changed from ribose to arabinose, glucose, phenyl, etc.

6,7-Dimethyl-9-(d-arabo)-isoalloxazine; 9-(d-arabo) isoalloxazine; 9-(d-gluco)-isoalloxazine; and 6,7-dimethyl-9-(d-xylo)-isoalloxazine were weakly effective. In the same series, 5,7-dimethyl-9-(l-arabo)-isoalloxazine; 9-(l-arabo)-isoalloxazine; 9-(ethoxy)-isoalloxazine; 3-methyl-9-(d-gluco)-isoalloxazine; 6,7-dimethyl-9-(d-gluco)-isoalloxazine; 3,6,7-trimethyl-9-(d-gluco)-isoalloxazine; tetramethylene-9-(l-arabo)-isoalloxazine; 6,7-dimethylisoalloxazine-9-acetic acid; isoalloxazine-9-acetic acid; 5,6-dibenzo-9-methyl-isoalloxazine; 9-phenyl-isoalloxazine; 6-methyl-9-(d-ribityl)-isoalloxazine; 1,2-dimethyl-4-amino-5-(d-ribityl)-aminobenzene; 1,2-dimethyl-4-nitro-5-d-ribityl-aminobenzene; and N-methyl-keto-quinoxaline carboxylic acid were all found negative as tested. In general, therefore modifications in the 9-position produced weak antagonists or compounds possessing no activity whatever.

In confirmation of the results of Kuhn et al. (1943), von Euler and Karrer (1946) prepared d-araboflavin and l-araboflavin and tested their effect on rat growth. d-Araboflavin was effective and the results were questionable with the corresponding laevo isomer.

Galactoflavin, one of the series of 9-substituted riboflavin compounds similar to those produced by Kuhn et al. (1943), was found by Emerson and co-workers (1945) to produce in the rat a riboflavin deficiency which could be prevented by riboflavin supplementation. For ratio of metabolite to displacer, the effect of 200 micrograms of riboflavin did not completely prevent the action of 2.16 mg. of the analogue.

Metabolite Analogues with Ring Structure Modification

The first and to date the only metabolite analogue of riboflavin to be prepared with a ring structure modification is 2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine (Fig. 2) which produces a riboflavin deficiency in bacteria (Woolley, 1944).

The dinitrophenazine from which this compound was prepared produced a mild riboflavin deficiency in mice. From his work with the phenazine and benzimidazole, Woolley suggested that benzene rings substituted for pyrimidine rings in biologically active compounds would produce drugs capable of displacing the corresponding substance. In the mouse, the results on growth indicate that it would require 100 mg. of riboflavin to counteract 200 mg. of the antagonist. Confirmation of Woolley's work on the diamino-phenazine comes from Sarett (1946) who found that the compound competitively inhibited the utilization of riboflavin and of flavin-adenine-dinucleotide by *Lactobacillus casei* and to the same degree.

Antimalarials as Metabolite Antagonists of Riboflavin

Riboflavin was reported by Madinaveitia et al. (1944) to inhibit the action of atabrine, quinine, propamidine, and methylene blue, using *Lactobacillus casei* as the test organism. Johnson and Lewin (1945) confirmed the inhibitory action of riboflavin for the antibacterial action of quinine.

In 1946, Madinaveitia published details of his earlier work which tended to show that substances active as antimalarials were antagonized by riboflavin, while those which were not antimalarials were not antagonized, when *Lactobacillus casei* was used as the test organism. Also in 1946, Hellerman and his group published work showing that quinine, atabrine, and other related substances inhibited d-amino acid oxidase strongly at low concentrations of flavin-adenine-dinucleotide and only slightly at higher concentrations. Nonantimalarial quinolines and other compounds also possessed this characteristic. The property of the compounds to combine with numerous proteins paralleled their action on d-amino acid oxidase and it was therefore suggested that competition existed for a surface position on the protein.

It was concluded from quantitative measurements that the activity of the molecules tested on protein had both a reversible and an irreversible phase. The irreversible phase probably underlay the results of Haas (1944) who found that the irreversible action of atabrine on cytochrome reductase was so marked that protection by riboflavin phosphate occurred only when it was added before the atabrine. Correlation of these findings with those of Sarett (1946), who reported stimulation by isoriboflavin of the utilization of riboflavin or flavin-adenine-dinucleotide in a bacterial system which was not adversely affected from the standpoint of growth, and inhibition by the

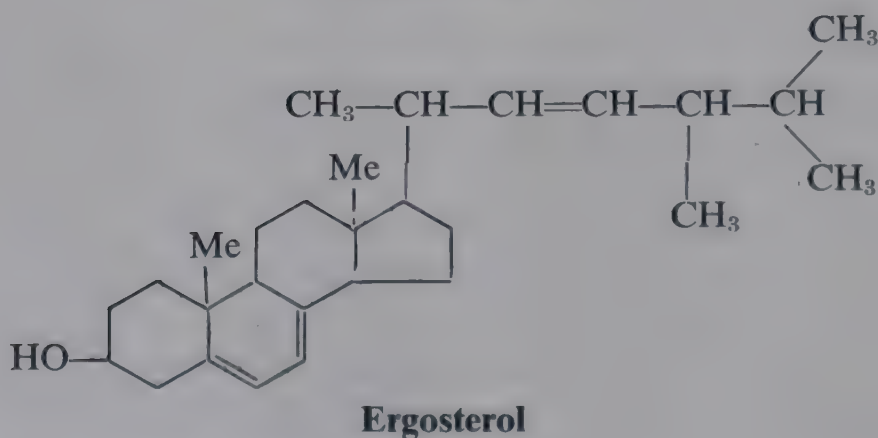
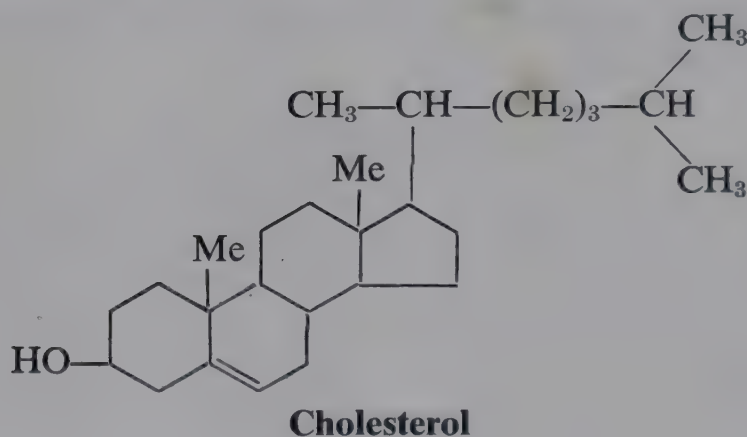
active ones in that they had a free hydroxyl group at C3, double bond between C5 and C6, and possessed a side chain.

With these findings under consideration, Fisher then determined the ability of these sterols to inhibit the hemolytic action of digitonin. Ponder (1945) had shown that the active point of digitonin fixation in the steroid molecule was the C3-C5-C6 region and Fisher found that the same sterol units which prevented hemagglutination were effective inhibitors of hemolysis by digitonin.

As previously mentioned the hemagglutination by influenza virus resembles that by *Hemophilus pertussis* supernatant. From the inhibition standpoint, however, the two are quite different. For example, the steroid inhibitors of the *Hemophilus pertussis* hemagglutination are ineffective against that of influenza virus and conversely the ovarian cyst polysaccharide which inhibits the virus hemagglutination is inactive against the pertussis reaction (Fisher, 1948). Again, erythrocytes treated with the receptor destroying enzyme of *Vibrio comma* remain agglutinable by pertussis supernatant but not by that of influenza virus.

Influenza Virus

Hirst (1942) from his studies of influenza virus adsorption on red cells concluded that the reaction between the virus agglutinin and the receptor at the cell surface was that of an enzyme-substrate interaction, and that the enzyme possessed by the virus changed the properties of the cell receptor



utilization of flavin-adenine-dimethylzide by *Lactobacillus casei* (Sevagi, 1944).

Recapitulation

Effective metabolic analogues of riboflavin have been produced by replacing the 6,7-dimethyl structure by 6,7-dichloro, shifting the 7-methyl to the 5-position, substituting a benzene ring for the pyrimidine ring, and replacing the 9-ribose with another similar sugar moiety. Certain analogues are effective in both bacterial and animal test systems, others, such as arabinoflavin, are not. Recorded results indicate the activity of certain metabolic antagonists of riboflavin as antimalarials, and yet they have never been tested as such. Pharmacological results with arabinoflavin demonstrated its activity.

While the picture is not as clear with riboflavin, it is probable that metabolic analogues of this vitamin have been or will be produced which prevent its synthesis, inhibit its incorporation into an enzyme system, and finally interfere with the activity of the preformed riboflavin-containing enzymes.

Here, again, the research still to be done in order to establish the utility of these agents far exceeds that now of record. Relatively few variations of riboflavin displacers have been synthesized, and these were apparently available in such small amounts as to block their general usage. The difficulty of synthesizing molecules of this type is a deterrent to many individuals and to most laboratories. It is apparent, however, that great discoveries await those with the perseverance and powers of application necessary for work in the field.

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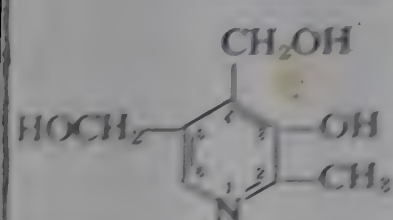
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Chapter 14

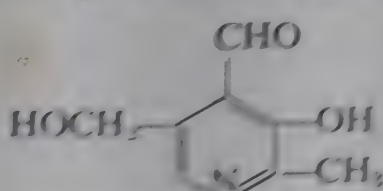
METABOLITE ANALOGUES OF PYRIDOXINE, NICOTINIC ACID, AND BIOTIN

Metabolite Analogues of the Vitamin B₆ Group

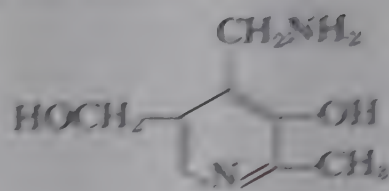


Pyridoxine

2-methyl-3-hydroxy-4,5-di-(hydroxymethyl)-pyridine



Pyridoxal



Pyridoxamine

1. Studies with Microorganisms.
 - a. Metabolite Analogues with Substituent Change.
 - b. Metabolite Analogues with Ring Change.
2. Studies with Animals, Chicks, and Human Beings.
 - a. Metabolite Analogues with Substituent Change.
3. Enzymatic Studies.
4. Chemotherapy and Pharmacology of Vitamin B₆ Metabolite Analogues.
5. Recapitulation.

The B₆ group of vitamins is broadly disseminated throughout the plant and animal worlds. There are bacteria which can synthesize pyridoxine and some which cannot. With some microorganisms specificity exists within the B₆ group in the sense that responses occur for example to two forms and not to the third. Pyridoxal phosphate is utilized by *Lactobacillus helveticus* and *Lactobacillus acidophilus* but not by *Lactobacillus casei*. Some bacteria utilize pyridoxamine phosphate more readily than pyridoxal phosphate. There are recorded instances of incapacity of bacteria to phosphorylate either pyridoxal or pyridoxamine. Deficiency of vitamin B₆ group in the rat is characterized by dermatitis, convulsive seizures, and impaired growth. Dogs, pigs, and chicks develop a hypochromic anemia on diets restricted with regard to these nutrillites. In the chick, α - and β -pyracin are biologically active.

It has been demonstrated by Scott and his associates (1945) that the

pyracins function in the utilization of folic acid conjugates by activating enzyme systems which liberate folic acid. This group of nitrilites probably bears a direct and immediate relationship to the essential fatty acids in that the two factors are mutually interchangeable in curing rat acrodynia. Human requirements for this factor were not established until the advent of the displacers which when fed to patients produced seborrheic skin lesions, cheilosis, glossitis, lymphocytopenia, and marked systemic manifestations with nausea, vomiting, weakness, and dizziness.

Gunsalus and his colleagues (1944) have established the biochemical functions of this group of molecules as being primarily associated with protein metabolism just as thiamine for example with carbohydrate metabolism. Pyridoxal phosphate is the coenzyme in the decarboxylation of tyrosine, arginine, and glutamic acids, as well as for the transaminase in the glutamate aspartate system; furthermore, it is essential for the synthesis of tryptophan from indole. Pyridoxal replaces cystine in the growth of certain microorganisms. It has been reported by Boyd et al. (1948) that *Clostridium perfringens* does not need lysine, alanine, aspartic acid, and glycine if pyridoxal or pyridoxamine is supplied. Further evidence of the importance of pyridoxine in protein metabolism comes from the demonstration that there is an increased requirement (for pyridoxine) when large amounts of glycine or of DL-serine are fed. Tryptophan metabolism is abnormal in the pyridoxine deficient animal as reflected in an increased urinary excretion of xanthurenic acid following the administration of the amino acid. In all of their enzymatic activities the phosphorylated forms of the B₆ group of vitamins are bound to protein. A final point concerning the enzymatic aspects of the B₆ group is that pyridoxamine phosphate as well as pyridoxal phosphate may function as a prosthetic grouping.

Pyridoxine is 2-methyl-3-hydroxy-4,5-di(hydroxymethyl)-pyridine and as such presents potentialities for the design of metabolite analogues in two major categories; substituent modification, elimination or replacement, and ring alteration. One point of major importance yet to be established is the phosphorylation site which was reported to be the 3-position by Karrer and Viscontini (1947, 1947a). Umbreit and Gunsalus (1949) have established the fact that tyrosine decarboxylase coenzyme is not pyridoxal-3-phosphate, which leaves positions 4 and 5 as probable.

Studies with Microorganisms

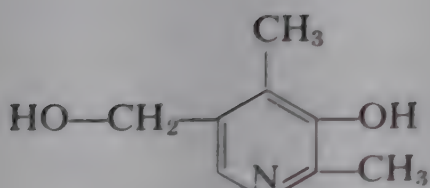
Metabolite Analogues with Substituent Change

The first metabolite analogue of the B₆ group of vitamins, desoxypyridoxine, was not tested by the original investigator (Ott, 1946) in bacterial systems. Martin et al. (1948) while investigating a new series of analogues checked the activity of desoxypyridoxine against *Saccharomyces cerevisiae*, G. M., and found it effective. Pyridoxal reversed its activity. Using the same

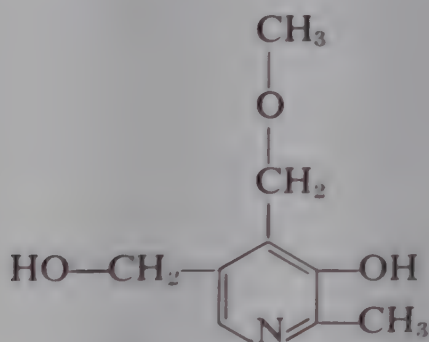
test organism, 2-methyl-3-hydroxy-4-hydroxymethylpyridine and 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine were found active as displacing agents for pyridoxine. The latter compound was a more powerful inhibitor than desoxypyridoxine. 2-Methyl-3-hydroxy-4-dimethylaminomethylpyridine was inactive.

Heyl et al. (1948) synthesized a series of pyridoxylamino acids by reductively coupling pyridoxal with amino acids and amino acid esters. There were 20 amino acids and four esters of amino acids converted into the corresponding pyridoxylamino acids, and not one of these possessed anti-vitamin activity when tested on *Lactobacillus casei*, *Streptococcus faecalis*, and *Saccharomyces carlsbergensis* (Snell and Rabinowitz, 1948).

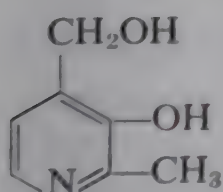
Irradiation of pyridoxamine under aerobic and anaerobic conditions



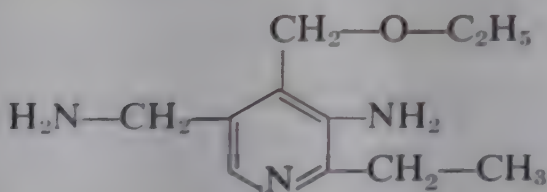
Desoxypyridoxine
2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine



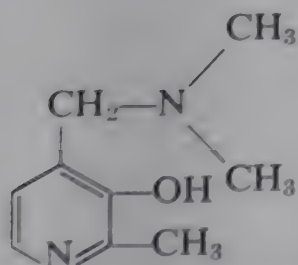
Methoxypyridoxine
2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine



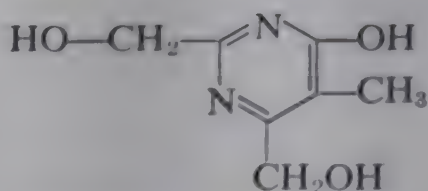
Desoxymethylpyridoxine
2-methyl-3-hydroxy-4-hydroxymethylpyridine



2-Ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine



2-Methyl-3-hydroxy-4-dimethylaminomethylpyridine



2,6-Di(hydroxymethyl)-4-hydroxy-5-methylpyrimidine

FIG. 1. Metabolite analogues of the vitamins B₆.

produces an antibacterial agent (Shwartzman and Fisher, 1947) which may well be a metabolite analogue of the nutilite with displacer capacity.

Table 1

METABOLITE ANALOGUES OF VITAMIN B₆ GROUP

<i>Name Metabolite Analogues with</i>	<i>Activity Substituent</i>	<i>Reference Modification</i>
Desoxypyridoxine	+	Ott, 1946; Martin et al., 1948
2,4-dimethyl-3-hydroxy-5-hydroxy-methylpyridine		
Methoxypyridoxine	+	Ott, 1947
2-methyl-3-hydroxy-4-methoxy-methyl-5-hydroxymethylpyridine		
2-Methyl-3-hydroxy-4-hydroxymethylpyridine	+	Martin et al., 1948
2-Ethyl-3-amino-4-ethoxymethyl-5-aminomethylpyridine	+	Martin et al., 1948
2-Methyl-3-hydroxy-4-dimethylamino-methylpyridine	—	Martin et al., 1948
Pyridoxylamino acids	—	Heyl et al., 1948; Snell and Rabinowitz, 1948
Irradiated pyridoxamine	+	Shwartzman and Fisher, 1947
<i>Metabolite Analogues with Ring Modification</i>		
2-Acetoxy-3,5-diacetoxymethyltoluene	—	Martin et al., 1948
2-Methyl-4-hydroxy-6-hydroxy-methylpyrimidine	—	McCasland et al., 1946
2,6-Di-(hydroxymethyl)-4-hydroxy-5-methylpyrimidine	—	McCasland and Tarbell, 1946

Metabolite Analogues with Ring Change

In the course of an investigation of possible antimalarials McCasland et al. (1946) synthesized 2-methyl-4-hydroxy-6-hydroxymethylpyrimidine and a number of related compounds. This compound apparently was not tested as an antimetabolite but the same group subsequently synthesized and tested 2-hydroxymethyl-4-hydroxy-5-methyl-6-hydroxymethylpyridine, which has the four characteristic groups of pyridoxine attached to a pyrimidine, instead of a pyridine, ring (McCasland and Tarbell, 1946). Using *Saccharomyces cerevisiae*, the compound was inactive. One other possible metabolite displacer with a ring modification and substituent change has been tested and found inactive. It is 2-acetoxy-3,5-diacetoxymethyltoluene.

Studies with Animals, Chicks, and Human Beings

Metabolite Analogues with Substituent Change

Only two analogues have been studied in vivo: desoxypyridoxine and methoxypyridoxine. Desoxypyridoxine was first studied by Ott (1946) as an antimetabolite, as previous studies had indicated that the molecule pos-

showed no vitamin activity (Unna, 1940). He conducted bioassays with chicks and found that, under the conditions of the experiments, two molecules of the inhibitor sufficed to counteract one molecule of pyridoxine. This ratio held for several levels of the vitamin and metabolite analogue. Studies of the histopathology of chicks and other animals (mice, rats, dogs, and monkeys) treated with desoxypyridoxine were undertaken by Muthett et al. (1947) who found atrophy and degeneration of the hematopoietic organs, particularly of the lymphoid series. On this basis, these investigators suggested the possible usefulness of this analogue in the treatment of tumors of the lymphoid tissue. Atrophy of the spleen occurred and in the thymus Hassall's corpuscles and the lymphoid tissue were involved. In general, the changes seen were similar to those observed in chicks and animals on pyridoxine deficient diets. In dogs, a microcytic anemia and a leukopenia developed within a month. From an examination of the adrenal glands in monkeys treated with desoxypyridoxine, it was considered possible that the lymphoid atrophy might be due to stimulation of the adrenals by the vitamin analogue. Comparison was not made among the various tissues of monkeys on a deficient diet with those receiving the analogue but gross changes were seen resembling those of pyridoxine deficiency; included were dryness of the hair, skin scaliness, tongue lesions, hyperirritability, and convulsions of epileptic nature.

Several studies have been made on the effect of desoxypyridoxine on the chick embryo. Cravens and Snell (1949) and Karnofsky et al. (1950) reached essentially similar conclusions regarding the action of desoxypyridoxine in this test system. In the 0 day egg, the analogue acted as a true displacer agent which could be counteracted by any of the three forms of the vitamin. After four days, the chick embryo became resistant to the displacer action of the molecule but susceptible to a direct toxic action which was not counteracted by any form of the nutritive. The proposal made by Cravens and Snell that the explanation of these findings lay in the phosphorylation of the analogue were rendered improbable by the observations of Karnofsky et al. who reported that pyridoxal phosphate did not counteract the toxicity of desoxypyridoxine in the 0 day egg as effectively as did pyridoxine. In the experiments of Cravens and Snell, nicotinic acid, nicotinamide, and DL-alanine proved ineffective in the counteraction of the inhibitory properties of desoxypyridoxine. This observation was interpreted to indicate that the inhibition of the conversion of tryptophan to nicotinic acid, which is known to be associated with the vitamin B₆ group, was not a factor in the results.

In the mouse, desoxypyridoxine exerts a powerful antivitamin effect, producing typical symptoms of vitamin B₆ deficiency (acrodyntia). The deficiency state is aggravated by high casein and by methionine. For mice receiving 2% pyridoxine per day, the inhibition index of desoxypyridoxine

was found to be 10. In the rat, Emerson (1947) observed the antagonistic action of the analogue when added to a pyridoxine deficient diet. The onset of symptoms was materially decreased and the symptoms of the depletion were aggravated. Later, Nelson and Evans (1948) reported marked reproductive upsets produced in normal adult female rats given desoxypyridoxine 10 to 20 days prior to breeding. Supplementation with pyridoxine on the day of breeding resulted in complete counteraction of the adverse effects of the antagonists. Fetal resorption generally characterized the reproductive upsets, although failure of implantation, stillborn young, decreased number of young per litter and decreased weight of the young at birth were also noted.

Until recently there had never been a clear-cut demonstration that pyridoxine deficiency states could be produced in man. The advent of the metabolite inhibitors offered the tool for a study of pyridoxine deficiency in the human being, and Mueller and Vilter (1949, 1950), recognizing this fact, used desoxypyridoxine to precipitate a deficiency syndrome in man which responded specifically to pyridoxine. Eight patients were given 60–150 mg. of the analogue daily while they were maintained on a diet low in vitamins of the B-complex. Within two to three weeks, seborrhea-like skin lesions developed about the eyes, nose, and mouth. Fifty per cent of subjects developed cheilosis resembling that of riboflavin deficiency and a glossitis which closely resembled that of niacin deficiency. Lymphocytopenia was noted. Complete cure of the syndrome resulted within 48–72 hours following the parenteral administration of 100 to 200 mg. of pyridoxine. The inhibitory ratio of antimetabolite to metabolite in these patients was estimated to be 1:1.

In 1940, Unna tested 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine (methoxypyridoxine) for vitamin activity in the rat and found it weakly active. When Ott (1947) tested this same molecule in the chick, he found it to be a potent antimetabolite. The ability of the rat to use methoxypyridoxine as a vitamin has been attributed by Porter et al. (1947) to conversion to pyridoxine. In Ott's experiments, the inhibition ratio was 4:1. Methoxypyridoxine seemed to possess an irreversible action in that when a lethal dose of the compound had been given, subsequent administration of pyridoxine was generally ineffective in preventing the death of the chick. On the other hand, the toxic action of desoxypyridoxine could be counteracted.

From the above, it was to have been anticipated that the methoxy analogue would be toxic to the chick embryo (Karnofsky et al., 1950). The toxicity decreases with the age of the embryo. Compared to desoxypyridoxine, methoxypyridoxine is at least 25 times as active as a vitamin antagonist in the 0 to four day old chick embryo. All forms of vitamin B₆ tested counteracted the methoxy compound; included were pyridoxine,

pyridoxal, pyridoxamine, and pyridoxal phosphate. Chick embryos treated with lethal doses of methoxypyridoxine continued to differentiate and emerge for the two to five day period before death. In view of the key position of pyridoxine in protein metabolism this is most unusual, however, the unrelated phenomenon of lack of specificity of pyridoxal phosphate as an inhibitor may explain the observation. It is, after all, pyridoxal phosphate which is the prime coenzyme in enzymatic processes associated with protein metabolism, and as this factor is not the key to the phenomenon of blockage here considered it must be assumed that pyridoxine itself plays the prime role in the chick embryo. There is clearly an intrinsic toxicity in all metabolic analogues which is dissociated from the capacity as an antimetabolite, and this factor will complicate such studies.

As might have been anticipated from the results of Uina (1947) with ethoxypyridoxine in the rat, the mouse is able to utilize this molecule as a pyridoxine precursor (DeRenzo and Cerecedo, 1950). This is by way of contrast with the efficacy of deoxypyridoxine in the mouse. In the studies reported by Mahieu et al. (1947), the histopathology produced by deoxypyridoxine and methoxypyridoxine in the chick was essentially the same. In dogs, while a single subcutaneous injection of 10 mg. per kilogram of the ethoxy compound produced convulsions and death, the blood values would indicate that the compound showed some degree of pyridoxine activity, reflected in a failure of the development of an abnormal blood picture. The deoxy molecule on the other hand produced microcytic anemia. There were other details of histopathology which indicated that at least in the dog the toxicity of the two analogues was not necessarily the same either from the quantitative or qualitative viewpoint.

Enzymatic Studies

The first approach to the study of the action of metabolite analogues of the vitamin B₆ group on enzymatic mechanisms came through the researches of Porter et al. (1947) who used metabolic processes in intact animals (rats). Pyridoxine plays a role in tryptophan metabolism which is reflected in the excretion of kynurenine in the normal animal and xanthurenic acid in the vitamin deficient animal. In all instances, deoxypyridoxine interfered with tryptophan metabolism and caused increased xanthurenic acid excretion. Methoxypyridoxine, on the other hand, acted under some conditions as an antimetabolite and under other conditions as a neutralizer. These authors concluded that the ether linkage in the methoxypyridoxine molecule can undergo cleavage to yield pyridoxine. One additional proof of the occurrence of such cleavage was the demonstrated increased pyridoxic acid excretion following administration of the methoxy molecule. As pointed out by Porter and his associates, the metabolic mechanism which they studied is extremely sensitive to the action of the antimetabolites as con-

trasted to the relative resistance of the rat itself to the development of the syndrome of B₆ deficiency following the administration of this same molecule.

Beiler and Martin (1947) investigated the action of desoxypyridoxine on tyrosine decarboxylase. The antimetabolite was ineffective in blocking the enzyme for which pyridoxal phosphate is the cofactor. Since Gunsalus, Bellamy, and Umbreit (1944) had shown that pyridoxal was not active as a coenzyme unless it was phosphorylated, these workers phosphorylated desoxypyridoxine and found the product to be an effective displacer for pyridoxal phosphate in the tyrosine decarboxylase system.

Confirmation and extension of the findings of Beiler and Martin (1947) came from the Merck Laboratories in 1949. From their observations, Umbreit and Gunsalus concluded that desoxypyridoxine exerts its inhibitory effect by first being converted to desoxypyridoxine phosphate, which then competes with pyridoxal phosphate for the apoenzyme. In the nonphosphorylated form, the desoxy analogue had no effect on tyrosine decarboxylase, aspartic-glutamic transaminase from heart muscle, or tryptophanase of *Escherichia coli*. Once pyridoxal phosphate is attached to the apoenzyme, desoxypyridoxine does not displace it. Furthermore, the antimetabolite does not interfere with the combination of pyridoxal phosphate with protein, nor does it prevent phosphorylation of pyridoxal by adenosinetriphosphate unless the amount of pyridoxal present is the limiting factor. Desoxypyridoxine phosphate would seem to be primarily inhibitory to the point of combination of pyridoxal phosphate with protein, although it does have some activity against formed tyrosine decarboxylase.

Chemotherapy and Pharmacology of Vitamin B₆ Metabolite Analogues

Studies of the effect of various chemical agents on the multiplication of T₂ *Escherichia coli* bacteriophage can be used as presumptive evidence of possible value in the chemotherapy of virus diseases. It was with this thought in mind that Wooley and Murphy (1949) ascertained the effect of desoxypyridoxine on bacteriophage. The molecule markedly inhibited multiplication under conditions which otherwise produced near maximum virus production. The action of the antimetabolite was reversed by pyridoxine, several fatty acids, glucose-6-phosphate, pyruvic acid, and less effectively by lactic, malic, fumaric, or succinic acids. This study should stimulate researchers to investigation of various pyridoxine displacers in virus chemotherapy.

Another report which points to future activities in this field is that of Stoerk (1947), who discovered the regression of lymphosarcoma implants in pyridoxine deficient mice. In view of his observation, it seems almost certain that metabolite analogues of pyridoxine will influence certain tumors.

Recapitulation

Studies of metabolite analogues of the vitamin B₆ group are complicated by the multiplicity of factors involved which include pyridoxine, pyridoxal, pyridoxamine, and α - and β -pyrazon as well as the mechanisms of inter-conversion of these factors with phosphorylation of pyridoxal and, finally, coupling of the phosphorylated vitamin with an apoenzyme. A metabolite inhibitor might act by preventing any single phase or combinations thereof leading to the final enzymatic form. It is further apparent from studies of the counteraction of the desoxy analogue by pyridoxal phosphate in the chick embryo that the vitamin B₆ group may act in some systems in a manner dissociated from the coenzyme function of pyridoxal phosphate. The intrinsic toxicity of some of the vitamin analogues not related to their antimetabolite capacity further clouds the picture.

There are reports of the antimetabolite activity of less than a dozen different molecular modifications of pyridoxine. All effective analogues are those with substituent modification; not a single inhibitor has been reported in which the pyridine ring has been replaced. In fact, only one effort was made toward this goal which resulted in the finding that the pyrimidine nucleus replacing the pyridine ring did not produce an antimetabolite.

The concept has been promulgated that with each B-complex factor there will be antimetabolites which prevent the formation of the factor, inhibit its conversion to an active enzyme form, and finally prevent the action of the reformed enzyme. In the instance of the vitamin B₆ group, no studies have been made with bacteria which possess the capacity to synthesize this factor. On the second phase, however, it has been established that desoxypyridoxine phosphate interferes with the attachment of the coenzyme, pyridoxal phosphate, to the apoenzyme which coupling would result in the formation of tyrosine decarboxylase. It has also been demonstrated that desoxypyridoxine phosphate inhibits the action of tyrosine decarboxylase.

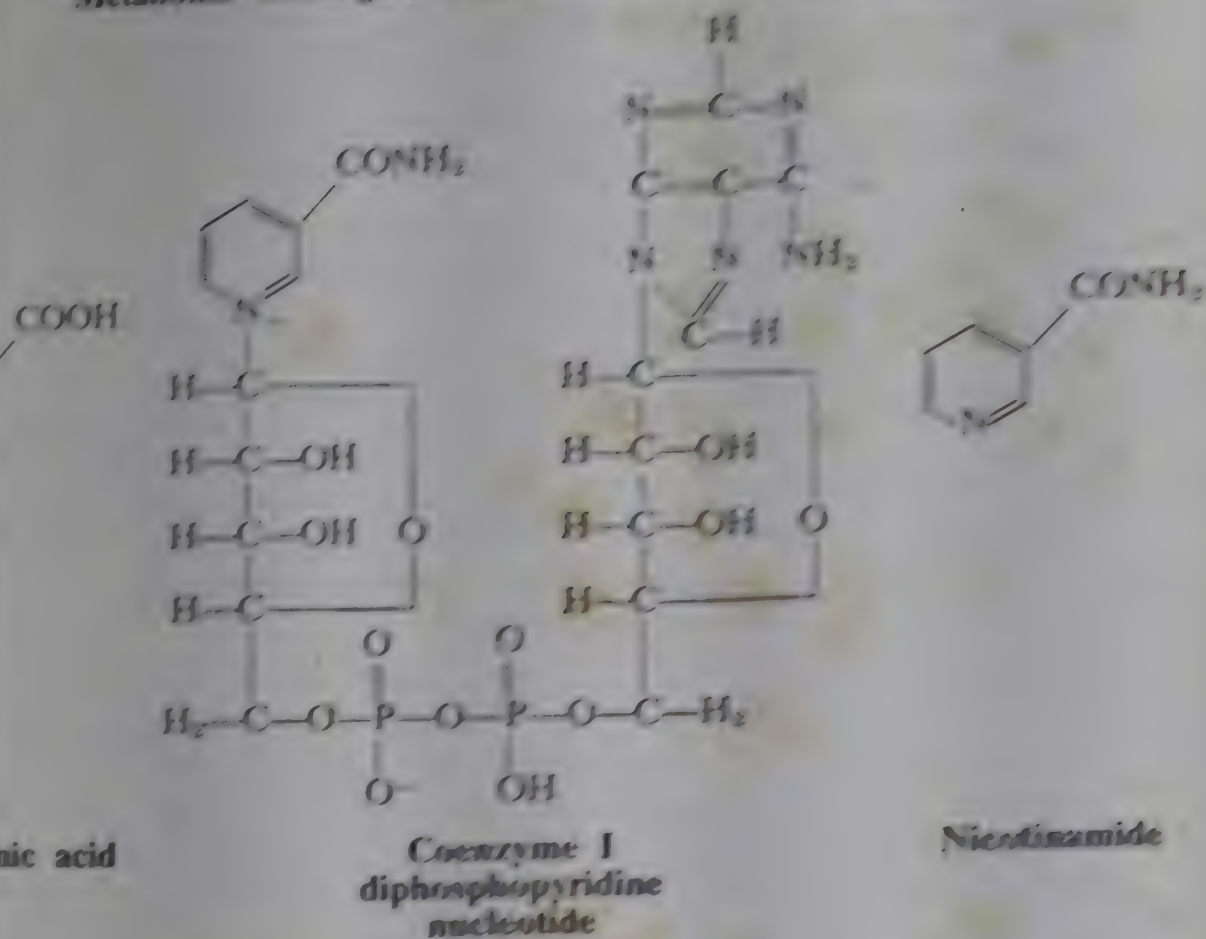
Methoxypyridoxine is an example of an antimetabolite effective in one system (the chick and chick embryo) and not in another (the mouse). This emphasizes the need of knowledge for metabolic transformation of the antimetabolite itself.

Extensions of study on the vitamin B₆ metabolite analogues will logically include consideration of the ability of D-alanine to replace vitamin B₆ phosphates. McNair and Snell (1950) have recently presented evidence of the duplication of the growth promoting action of natural materials in certain bacterial systems by pyridoxamine phosphate, thymidine or other desoxyribosides, vitamin B₁₂, and certain reducing agents. This work adds fuel to the concept that metabolite analogues will not reach maximum effectiveness unless and until they are used in combinations.

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Metabolite Analogues of Nicotinic Acid and Amide



Studies with Microorganisms.

a. Analogues with Substituent Modification.

b. Analogues with Ring Modification.

Animal Studies.

Enzymatic Aspects.

Chemotherapy and Pharmacology.

Recapitulation.

The curative factor for pellagra, nicotinic acid, was known as a chemical factor 75 years before its vitamin activity was discovered; and now, some 12 years after the establishment of this factor and its nutritional role, pellagra is still widespread in the United States. Experimental work leading to the isolation and identification of the nutriline was associated with studies of pig and chick blacktongue and, of course, with human pellagra. Nicotinic acid is known to occur in all living cells and to be an essential factor for plants, microorganisms, chicks, pigs, dogs, human beings, and probably rats. There are bacteria capable of synthesizing their own nicotinic acid, those, including *Staphylococcus aureus*, *Bacillus diphtheriae*, etc., which are dependent upon exogenous sources of supply of the nutriline, and finally parasitic microorganisms such as *Bacillus influenzae*, which need an external supply of the coenzymes for normal growth. Deficiency of nicotinic

acid in the human being causes pellagra, which is characterized by glossitis, dermatitis, diarrhea and other disturbances of the gastrointestinal tract, mental disorders and lesions of the central nervous system.

Free nicotinic acid does not occur in living tissues; nicotinamide and the enzyme forms are found. The nicotinamide-containing enzyme systems are primarily dehydrogenases for hydrogen transport; two coenzymes of this class of dehydrogenases are known as coenzyme I and II, also called diphosphopyridine nucleotide and triphosphopyridine nucleotide. In the body, the white cells of the lymphoid and myeloid series seem capable of converting nicotinic acid into the coenzyme forms which appear interconvertible by most living tissues. The ring nitrogen of the nicotinamide in the coenzyme is written in the form of a quaternary pyridinium salt, which in the dihydro-form possesses an additional acidic group. Magnesium ions are essential to the activity of these anaerobic dehydrogenases. Animals can utilize the coenzyme forms but it is not known whether they are broken down and resynthesized or absorbed intact.

Nicotinic acid and its metabolic end-products, nicotinic acid, trigonelline, and N'-methyl nicotinamide are excreted through the urine. The vitamin functions in protein and carbohydrate metabolism through its enzymatic hydrogen-transporting forms. Presumptively, there are functions of nicotinic acid which cannot be explained as operating via the coenzyme forms; i.e., blacktongue in dogs is cured more readily by nicotinic acid than by a corresponding amount of cozymase.

The discovery that a diet containing corn tends to precipitate pellagra led to the observation of the interrelationship of nicotinic acid and tryptophan (the amino acid may be the precursor of nicotinic acid) which is controlled by pyridoxine.

Presence of a water-soluble pellagrigenic agent in corn was proposed but recent opinion inclines to the view that amino acid imbalance or carbohydrate component could explain the underlying experimental observations.

As a model of metabolite analogues, the nicotinic acid or nicotinamide molecules might be changed as to ring and substituent. The coenzyme forms offer multitudes of potentialities for modification, but complexities of synthesis would limit this approach.

Studies with Microorganisms

Analogues with Substituent Modification

Mellin (1940) made the first of his many outstanding contributions to the study of metabolite analogues when he designed pyridine sulfonic acid amide (Fig. 1) which resembled nicotinic acid as sulfanilamide did p-aminobenzoic acid. His extensive investigations covered not only pyridine sulfonamide but the corresponding acid as well, and the interaction of these analogues with three forms of the nutritive: nicotinic acid, nicotinamide

and coenzyme I (cozymase) in three bacterial test systems. The importance of his report justifies the inclusion of Table II detailing results obtained.

As Table II shows, when *Staphylococcus* was the test organism pyridine sulfonamide inhibited nicotinamide-promoted growth more than it did that of nicotinic acid. With this organism therefore, inhibition and type of inhibition depends on the inhibiting and growth promoting agents present.

Table II*

SUMMARY OF TYPE AND EXTENT OF INHIBITION†

Promoter (Nicotinic Acid, Amide, or Cozymase)		Inhibitor (Pyridine Sulfonic Acid or Its Amide) 10 ⁻²		Type	
		ret.	red.		
<i>Staphylococcus</i>					
Acid,	10 ⁻⁵ —8 × 10 ⁻⁸	Amide	0	1/5—1	(I)
Amide,	10 ⁻⁶ —10 ⁻⁷	"	0	1/25	I
Cozymase,	10 ⁻⁷	"	1	1/5—1/25	II
	2 × 10 ⁻⁸	"	4—5	1/5—1	
	4 × 10 ⁻⁹	"	> 5		
Acid,	10 ⁻⁶	Acid	1—2	1	II
	2 × 10 ⁻⁶	"	3	1/5	
	4 × 10 ⁻⁷	"	5	1/5	
Amide,	2 × 10 ⁻⁷	"	4	1/5	II
	4 × 10 ⁻⁸	"	> 6	..	
	8 × 10 ⁻⁹	"	> 6	..	
Cozymase,	10 ⁻⁶	"	..	..	II
	2 × 10 ⁻⁷	"	..	..	
	4 × 10 ⁻⁸	"	..	..	
<i>Proteus</i>					
Acid,	5 × 10 ⁻⁷ —2 × 10 ⁻⁸	Amide	< 1	1	(III)
Amide,	2 × 10 ⁻⁷	"	2	1	II
	4 × 10 ⁻⁸	"	3	1	
	8 × 10 ⁻⁹	"	> 4	..	
Cozymase,	5 × 10 ⁻⁷	"	> 1	1	(II)
	10 ⁻⁷	"	1	1/5—1	
Acid,	10 ⁻⁵ —8 × 10 ⁻⁸	Acid	2	1	III
Amide,	10 ⁻⁵ —10 ⁻⁷	"	0	1	None
Cozymase,	10 ⁻⁵ —8 × 10 ⁻⁸	"	> 6	..	III
<i>Coli</i>					
0		Amide	1	1	
Amide,	10 ⁻⁶	"	0	1	
0		Acid	> 6	..	
Acid, amide, . or cozymase	10 ⁻⁵ —2 × 10 ⁻⁶	"	> 6	..	

* From Henry McIlwain, *Brit. J. Exper. Path.*, **21**, 136, 1940.

†ret. = Retardation of growth, expressed in days.

red. = Reduction of growth, expressed as the fraction of growth promoter actually present to which the inhibited growth corresponds. Absence of reduction is thus given by the figure 1.

Concentrations are expressed in molarities. The highest concentration of promoter in each set is approximately the optimal concentration for normal growth.

Type of inhibition is explained on p. 314 in the text. When the inhibition is not sufficiently large to be well characterized, the probable type is given in parentheses.

Inhibition is also profoundly affected by concentration. Using *Proteus*, for which nicotinic acid is vital, the sulfonamide inhibited all three types of growth factor: nicotinic acid, nicotinamide, and coenzyme I. Pyridine sulfonic acid inhibited nicotinic acid-promoted growth, but with nicotinamide present the compound actually accelerated growth. *Escherichia coli*, requiring no nicotinic acid for growth, was inhibited by either the sulfonamide or the sulfonic acid in high concentrations, but this action was not reversed by any growth factor. From these and other results, McIlwain postulated these types of inhibition:

Type I: This is comparable to competitive inhibition, in that the growth promoter and the inhibitor compete for the same reaction site on the enzyme surface. Inhibition is manifested as reduction in growth; there is no retardation. In a consideration of the problem at hand, it must be recalled that the inhibitors are interfering with a series of reactions and not with a single mechanism; nicotinic acid is converted in the organism into molecules of at least the complexity of pyridine nucleotides. Furthermore, any given cell is capable of adapting itself to changing environment. In this Type I inhibition, the effect is greater the higher the concentration of inhibiting agent and the lower the concentration of substrate. Type I inhibition is exemplified by the interrelationships of nicotinamide and pyridine sulfonamide where *Staphylococcus* is the test organism.

Type III: This is noncompetitive for here the inhibitor is adsorbed at a different position on the enzyme surface. Inhibition depends only on inhibitor concentration and does not vary with substrate concentration. Growth is retarded but there is no reduction. This is seen using *Proteus* as test organism with pyridine sulfonic acid as inhibitor and nicotinic acid as growth promoter.

Type II: This is a superposition of competitive and noncompetitive inhibitions; a combination of types I and III. The pyridine sulfonic acid inhibition of nicotinic acid or nicotinamide-promoted growth of *Staphylococcus* is given by McIlwain as an example.

It can be said that McIlwain first utilized the concept of inhibition analysis. He predicted that nicotinamide was not used by *Proteus* solely for the production of coenzyme I, basing this upon the observation that pyridine sulfonic acid inhibited coenzyme I growth promotion but did not interfere with that of nicotinamide.

Slightly different results were obtained by Erlenmeyer et al. (1942) who reported that pyridine-3-sulfonic acid did not inhibit growth of *Staphylococcus aureus* either with or without added nicotinamide. On the other hand, they confirmed McIlwain's results with pyridine sulfonamide. 2-(Pyridine-3-sulfonamide)-pyridine was a growth factor and not an inhibitor. This latter molecule had been prepared by McIlwain (1940a) and found less active than either pyridine sulfonic acid or the amide as an inhibitor of

bacterial growth. The reason for the conflicting result is not apparent. Pyridine sulfonic acid has been reported by other investigators to be an inhibitor of bacterial growth (Adler et al., 1943; Dreizen et al., 1948).

Möller and Birkofer (1942) extended the list of nicotinic acid displacers adding picolinic acid and α -picoline. This same group (Möller and Birkofer, 1942a) found that the inhibitory action of pyridine- β -sulfonamide was reduced or eliminated by nicotinic acid, nicotinamide, thiazole-carboxylic acid, coenzyme I, and by salts of zinc, manganese, copper, and iron, when *Streptobacterium plantarum* was the test organism. Due primarily to the inorganic ions which were effective this list seems extensive beyond reason from the metabolite analogue standpoint. The implication here is that chelation might offer an explanation. It is known that magnesium and manganese aid the activity of coenzyme I. The other metals which were active might well be functioning as cofactors in enzymatic systems converting one nicotinic acid form into another.

Several molecules related structurally to nicotinic acid have been reported inactive in bacterial systems against nicotinic acid stimulated growth. These include 3-acetylpyridine (Woolley, 1945), bisnicotinamide (Gautier and Cordier, 1947), and nicotinamidine (Barber and Slack, 1944). In

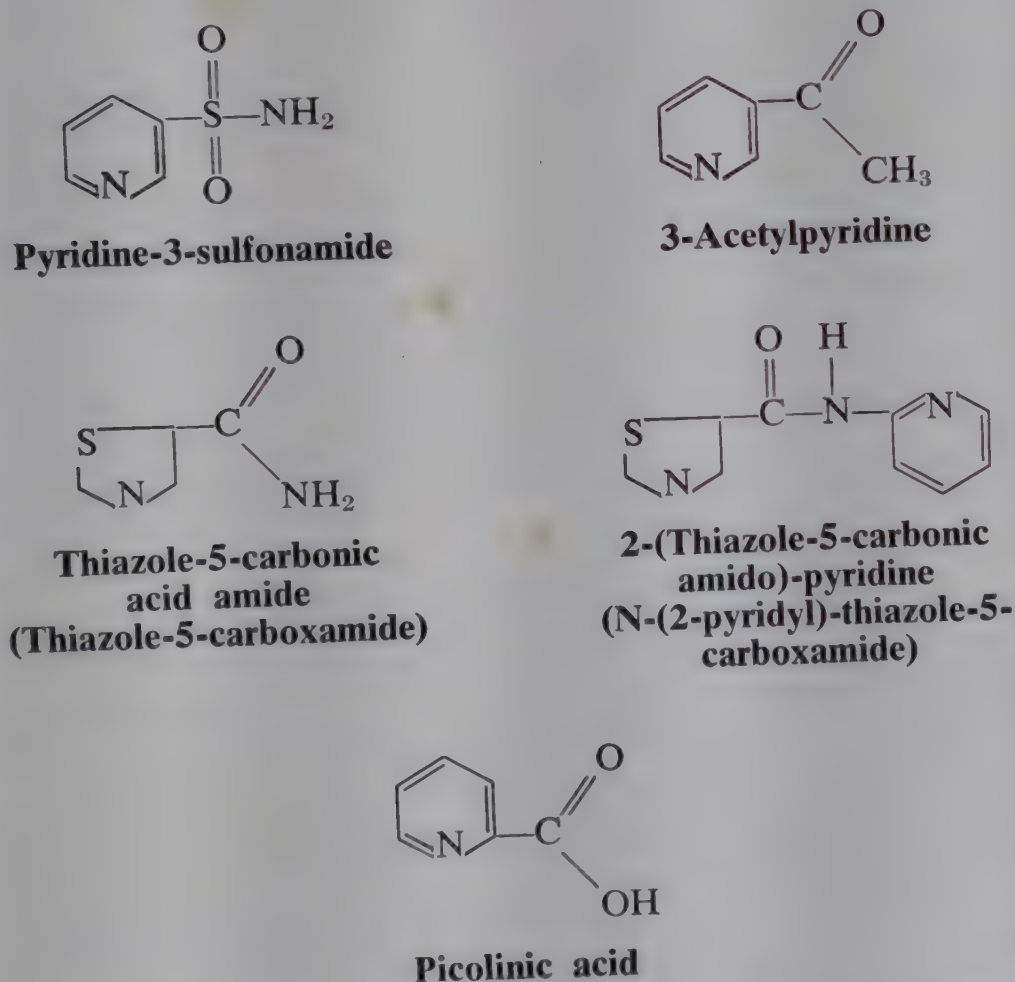


FIG. 2. Metabolite analogues of nicotinic acid—nicotinamide.



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